

Investigating Heparin Dynamics through Solid-State Nanopores: toward nanopore-based methods for glycomics

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Summary

Solid-state nanopores have become a fast-rising technique in single-molecule sensing, especially in DNA and protein sensing. Carbohydrates, as one of the most important biomacromolecules for human life, have posed challenges in related research and analysis due to their complexities, and solid-state nanopores have shown great potential in glycan sensing and analysis because of their stability, cost-effectiveness, fast results and high sensitivity.

The kinetics, conformation, and identification of heparin molecules were investigated. Heparin is a widely used anticoagulant in medical practices, and a large-scale contamination scandal in 2008 has attracted more attention, as a quick and effective method to differentiate heparin and OSCS (oversulphated chondroitin sulphate) needs to be developed. A series of experiments was done in different salts and salt concentrations, at different pH levels, to find an optimized sensing condition for maximum capture. By a simulation tool developed in the lab and the experimental results, we also investigated conformations of heparin as they pass through a solid-state nanopore. However, the negatively charged small particles pass through the nanopores too fast for the state-of-the-art instruments to fully resolve. An attempt was also made to differentiate heparin and OSCS, but no significant differences were seen.

In an effort to slow down the molecules, a specialized flow cell that incorporates a pressure system was adopted. However, under a small counterpressure, heparin molecules were not seen to slow down. Lectin, a human protein that has proved to be heparin-binding, was also experimented with. It was shown that at 3.6 M LiCl, pH 4, heparin and lectin molecules could bind and form a compound that translocates through the nanopore as one, which produces significantly slower and deeper signals.

Acknowledgements

I wanted to first express my gratitude to my supervisor, Vincent Tabard-Cossa. Thank you for the opportunity you have given me to get a glimpse of the nanopore world. But more importantly, I wanted to thank you for the support and empathy you have shown me and everyone in the lab, which was something so meaningful and very rare for me during my undergraduate years, and I will always appreciate.

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Furthermore, I wanted to extend my gratitude to every professor who taught me here at the University of Ottawa. You have all shown me a great learning experience and kindness that I will always remember.

Thank you to Dr. Janelle Sauvageau and Parsa, who both helped a great deal in our collaboration and the writing and publication process of the paper.

Lastly, I have infinite appreciation for my parents, who sent me to start my Grade 10 here in Ottawa, exactly 10 years ago. I would never be here without your hard work and unconditional support.

Statement of Originality

The material presented in this thesis results from research performed under the supervision of Dr. Vincent Tabard-Cossa in the Department of Physics at the University of Ottawa.

Chapter 2 contains a manuscript submitted as a first author (currently at the 2nd revision stage at Electrophoresis), in collaboration with Dr. Janelle Sauvageau and Mohammad P. Jamshidi from the National Research Council of Canada, and Dr. Kyle Briggs and Matthew Waugh from the T-Cossa lab. I performed all experiments and analysis included in this chapter except the data presented in Figures S4 and S5 (Calibration curves of dextran ladder and size exclusion HPLC traces of dextran ladder, heparin, and OSCS).

Chapter 3 contains one experiment (heparin with counter pressure), which was designed and performed by a PhD student in the group, Mohamed Yassine Bouhamidi, under the supervision of Dr. Vincent Tabard-Cossa. I analyzed this data set. The other experiments shown were performed and analyzed by me.

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1. Chapter 1. Introduction

1.1. Nanopores

Nanopores are small holes in a thin membrane of sizes in the nanometre scale. When immersed in solution and an external voltage is applied, they can be used as single-molecule detectors. As the target molecule is driven through the pore by the external voltage, it partially blocks the current trace, and we can observe its translocation as a current block. Figure 1.1 a shows the translocation of a single heparin molecule, and b shows the traces of heparin molecules passing through a solid-state nanopore. From analyzing these signals, not only do they confirm detection and reveal detection and other limits, but we can also gather information on molecular dimensions, conformations, charge state, and kinetics of these translocations.

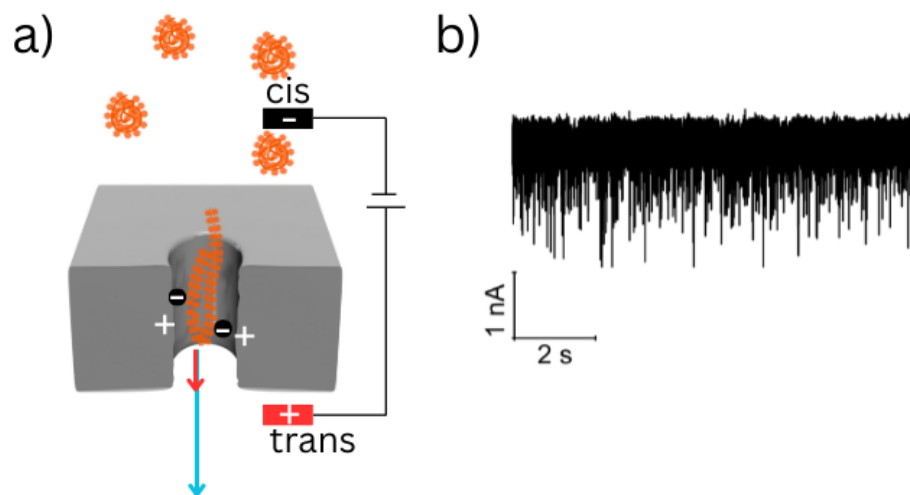


Figure 1.1 Nanopore sensing basics. a) Translocation of a heparin molecule through a solid-state nanopore. b) 10-second trace of heparin molecules passing through a ~5 nm silicon nitride solid-state nanopore, in 4 M KCl solution at pH 4.

Generally, there are two types of nanopores: biological and solid-state nanopores. Biological pores are openings embedded in lipid bilayers, and they were the first to be utilized for DNA detection. In 1996, Kasianowicz et al. were able to observe ssDNA translocations through a biological nanopore, which was made by α -hemolysin, a pore-forming protein[1].

Over the years, various types of engineered biological pores, including different proteins, peptides, and DNAs, have been utilized for applications across fields[1,2]. Figure 1.2 showcases single molecules passing through two different biological nanopores. However, biological nanopores have limitations, as they depend on the properties of the pore-making agents. Many efforts have been made to optimize sensing in biological pores, for example, engineering pore geometry for a more suitable sensing length and developing coatings to increase stability[3–5]. However, the disadvantages of biological pores remain. Biological pores mostly have a diameter of 1 to 4 nm, making them suitable for single-molecule detection like ssDNA, but limiting the detectable molecules, since larger molecules cannot pass through. These pores also have a shorter lifetime, typically hours, due to the fragility of the supporting lipid bilayer, although Oxford Nanopore Technologies has developed polymeric membranes to commercialize their biological nanopore sequencing platforms[6,7]. The operating range, in terms of temperature, voltage, solute and solvent type, is also somewhat limited, or else the pore sensing performance degrades (e.g., it gates, or becomes noisy). In order to expand the types of molecules captured by nanopores and optimize detection under various conditions, many have turned to solid-state nanopores for a more versatile solution.

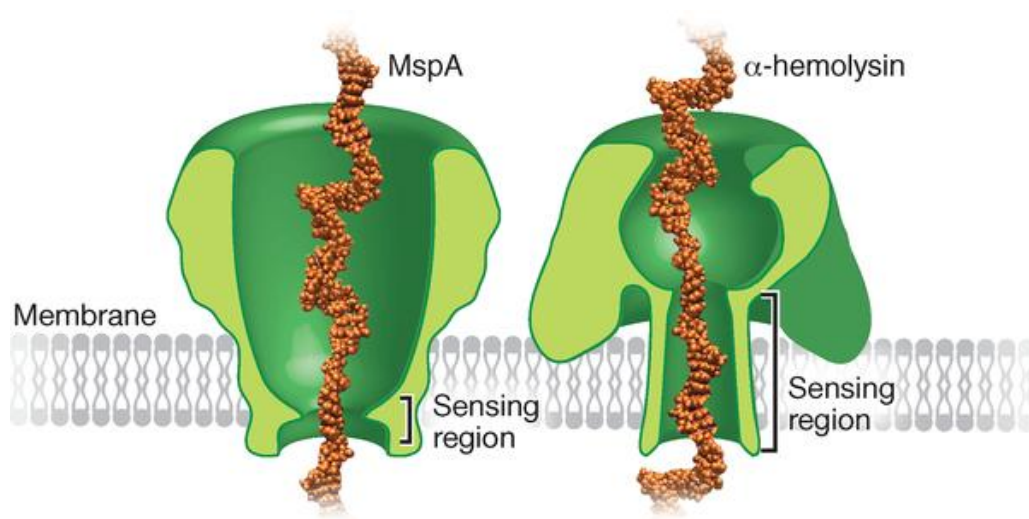


Figure 1.2 Molecules passing through MspA and α -hemolysin biological nanopores. From *Three decades of nanopore sequencing*, Deamer et al., 2016. Copyright Nature Biotechnology 2016, reprinted with permission.

Solid-state nanopores have become popular in recent years because of their stability and availability for diverse functionalization, as a bigger pore and superior chemical resistance have made many modifications possible, compared to their biological counterparts[7]. Many materials have been found adequate for the thin membrane. Silicon, metal (aluminum, hafnium), and other 2D materials like graphene are amongst the common choices for solid-state nanopores[8,9]. While biological pores use a transmembrane material that already has a nanopore structure, solid-state nanopores need to undergo fabrication, where a nanoscale pore is installed on the thin membrane. One straightforward way proposed is to use transmission electron microscopy to produce a powerful and extremely concentrated electron beam to drill a nanopore directly on the membrane. This method is successful in making a nanopore as small as 1 nm, but its high cost raises questions about accessibility[10].

Another more cost-effective method that has become popular in recent years is controlled dielectric breakdown[10]. The membrane is immersed in solution, and a pair of electrodes is used to apply an external electric field. Under a high enough voltage, the membrane goes through dielectric breakdown, and a nanopore is formed. Figure 1.3 illustrates these two methods for solid-state nanopore fabrication.

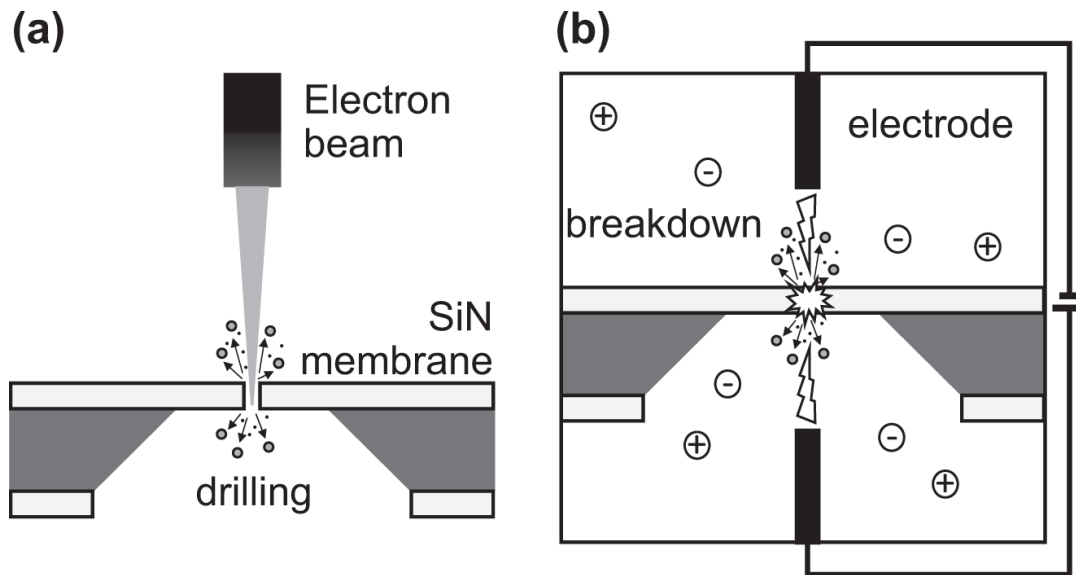


Figure 1.3 Different nanopore fabrication techniques. **a)** fabricating a nanopore on a silicon nitride membrane by using a direct electron beam to drill a hole through the membrane. **b)** fabricating a nanopore on a silicon nitride membrane by controlled electric breakdown. From *Solid-state nanopores towards single-molecule DNA sequencing*, Goto et al., 2020. Copyright Journal of Human Genetics 2020, reprinted with permission.

Solid-state nanopore as a tool for single-molecule detection and sequencing has been most well established for DNA[11]. As DNA molecules enter the access region, in order to pass through the nanopore, the coil is linearized and threaded through the pore. This unique process of local linearization during nanopore translocation offers valuable insights and information, as all segments of the molecule are now visible to the nanopore. Figure 1.4 shows the DNA translocations. Solid-state nanopores have opened the doors to a faster, more accurate and cost-effective method for DNA sequencing.

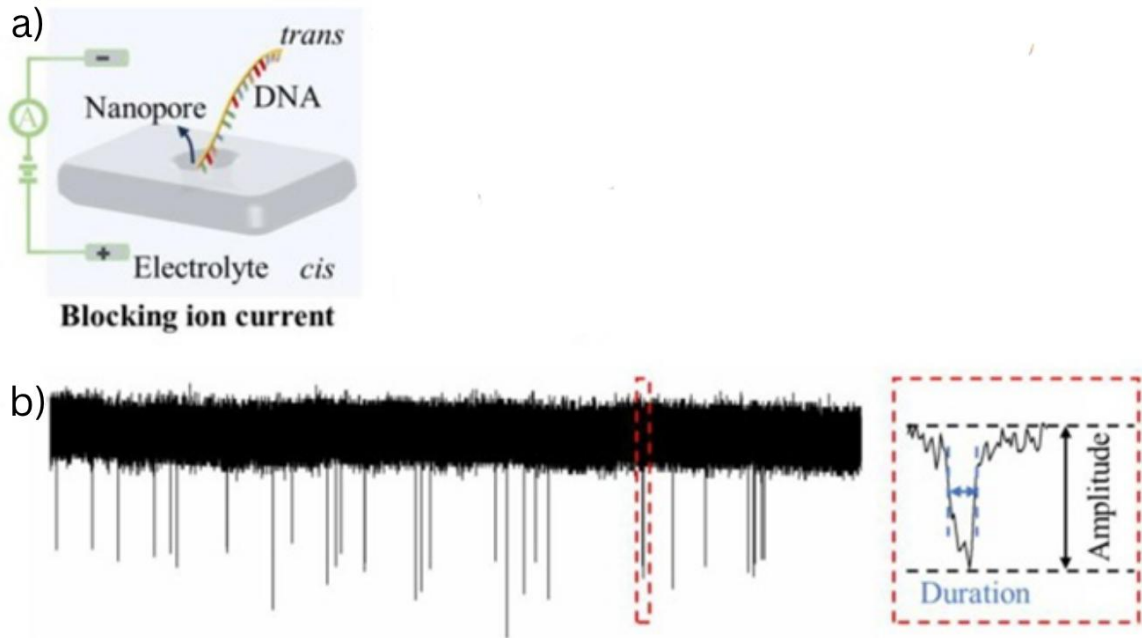


Figure 1.4 DNA sensing with solid-state nanopore. **a)** Schematics of a DNA translocating through a solid-state nanopore. **b)** traces and an individual event of DNA translocations. From *Solid-State nanopore DNA sequencing: Advances, challenges, and prospects*, He et al., 2024, Copyright Coordination Chemistry Reviews 2024, reprinted with permission.

As the potential of solid-state nanopores in analyzing DNA quickly attracts attention, the possibility for other biomolecules has also been explored. Proteins are important macromolecules in understanding many biological processes and diseases, and studying them at a single-molecule precision has been difficult because of their small size and complex structures[12]. Solid-state nanopores have made this possible, as the precise nanoscale pore size allows proteins to pass through individually. By analyzing the signal duration, depth, and event shape, solid-state nanopores can also offer information on high-accuracy protein sizing, conformation, and even identification[13]. Figure 1.5 shows an example of protein identification with solid-state nanopore systems.

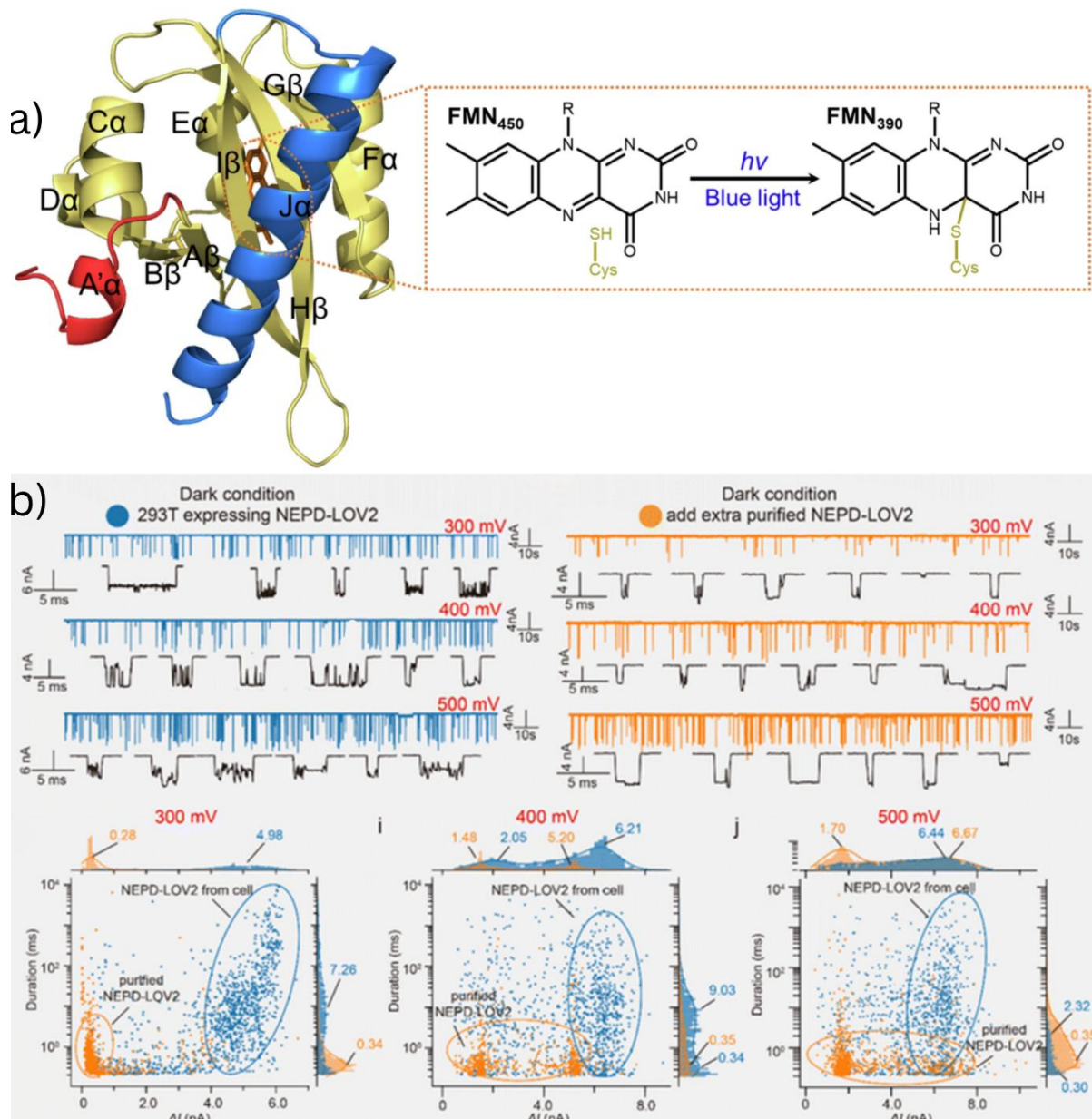


Figure 1.5 Structure and nanopore signals of LOV2 protein. a) Structure of the LOV2 protein, figure from *Structural dynamics of protein-protein association involved in the light-induced transition of Avena sativa LOV2 protein*, Kim et al., 2024, licensed under CC BY 4.0; b) representative traces of NEPD-LOV2 proteins extracted from cells versus purified at different voltages. Scatter plots and histograms showing distinct populations for NEPD-LOV2 proteins extracted from cells versus purified. From *High Accuracy Protein Identification: Fusion of Solid-State Nanopore sensing and Machine Learning*, licensed under CC BY-NC-ND 4.0.

Solid-state nanopores have also gained the attention of the glycomics and carbohydrate community. Similar to proteins, many sugar molecules are very small and exhibit complex chemical structures and properties. Solid-state nanopores as single-molecule detectors have

also provided many insights into their molecular size, conformation, and kinetics. Many have attempted to achieve tag-free identification of glycans through solid-state nanopores and seen some success[14–16]. Figure 1.6 shows glycan traces through solid-state nanopore systems. However, small molecules can translocate at an extremely high speed, often in a 0.1 to 10 μ s range, and given the limited resolution, even with state-of-the-art technology with a $\sim$ 1 MHz bandwidth, the information we can extract is incomplete. To address these difficulties, many ideas have been proposed, including modifications of the pore or molecular tagging and fragmentation[17,18].

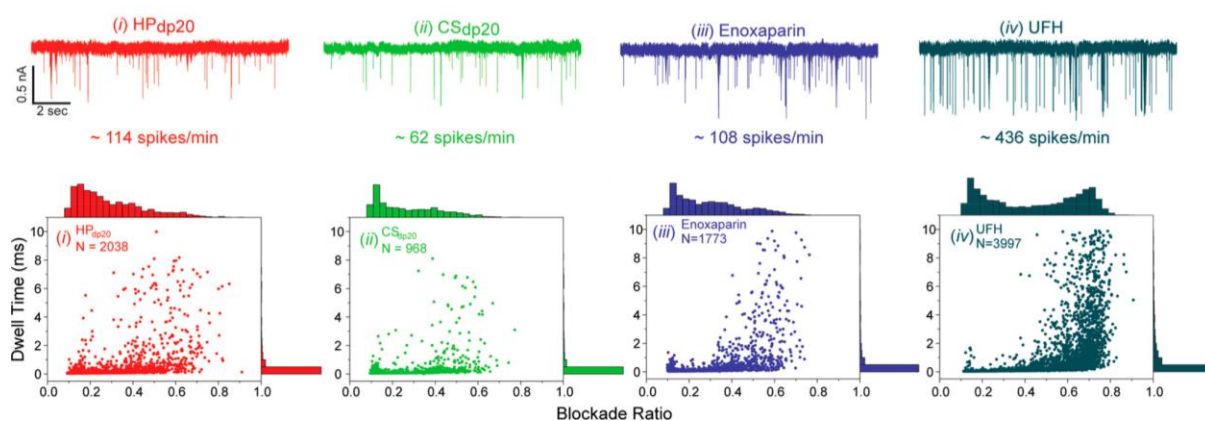


Figure 1.6 Representative traces and population of different sugar molecules (i: heparin, ii: chondroitin sulphate (CS), iii: enoxaparin, iv: UFH (unfractionated heparin)) translocating through a solid-state nanopore. From *Single Molecule Identification and Quantification of Glycosaminoglycans Using Solid-State Nanopores*, Im et al., 2019, Copyright ACS Nano 2019, reprinted with permission.

1.2. Nanopore Fabrication and Enlargement

5 mm x 5 mm chips with 200 μm -thick silicon frames purchased from Norcada are used most commonly as our nanopore supplies. The single membrane (1X) has a 40 μm x 40 μm area containing a free-standing Silicon nitride (SiN) membrane with an oxide layer and a thickness of 12 nm $\pm$ 2 nm. Figure 1.7 demonstrates the chips we use. More recently, 4-membrane (4X) and 8-membrane (8X) custom chips from Norcada and made in-house were also incorporated into our workflow. Since there are multiple individually addressable nanopores on a single chip, these devices allow for a higher experimental rate of low-noise nanopores that can successfully pass analytes.

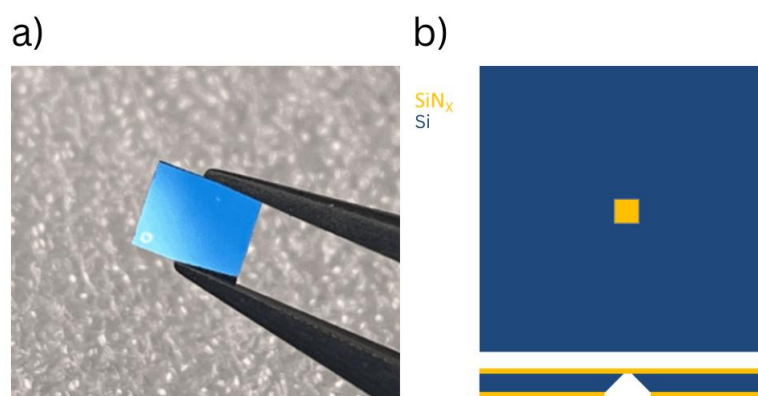


Figure 1.7 Silicon nitride membrane chip. a) photo of a Norcada silicon membrane chip. b) schematics of the membrane chip showing the silicon and silicon nitride layers and the membrane window.

Flow cells for 1X chip were designed and 3D printed in the lab, where two fluidic cells were equipped to allow the chip to have access to the solution, and a pair of rubber gaskets (5.6 mm x 5.6 mm with a 1.7 mm x 2.6 mm cut-out in the centre) was used to reduce noise by limiting the contact area of the chip with the solution. Figure 1.8 shows schematically the structure and assembly of a 1X flow cell and fabrication unit.

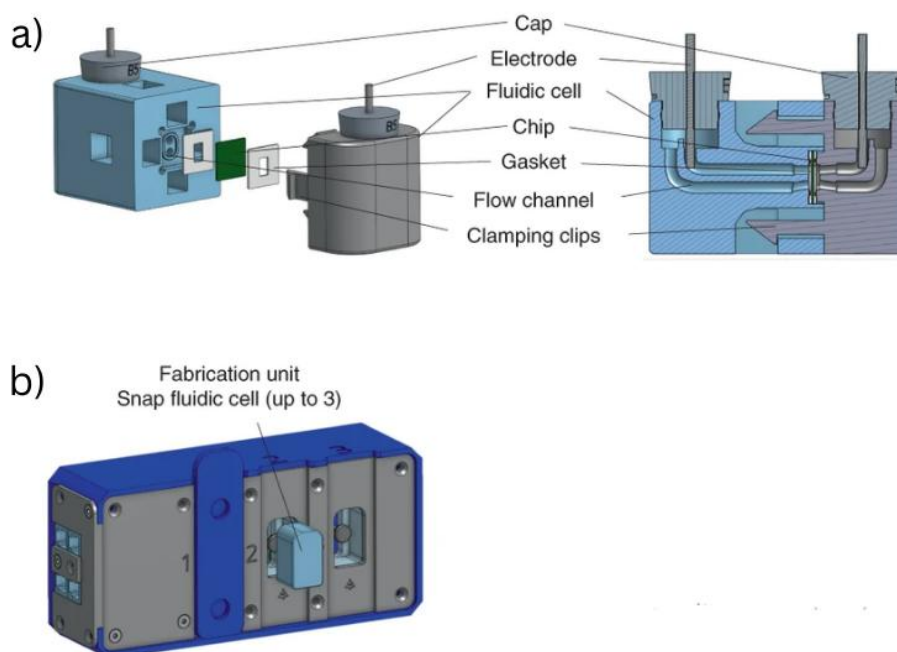


Figure 1.8 Structure of a flow cell and the fabrication unit. a) schematics of an assembled flow cell we use for the chip. **b)** schematics of inserting the flow cell into a fabrication unit.

After the chip and the flow cell were assembled, we first flushed 200 μL of isopropyl alcohol with a pipette into both sides of the fluidic cell. The use of IPA here is to clean the chip and ensure proper wetting. Then, as our established practice for best results, another 600 μL of 1M KCl at pH 8 (buffered with HEPES) was flushed into the cell from both sides. Now, we make sure the chip has proper contact with the solution by connecting the electrodes to a resistor. If the resistance shows a sudden increase, we can put the flow cell into a fabrication unit, where we would fabricate the nanopore with controlled dielectric breakdown.

In our lab, an automated CBD method is deployed to fabricate a nanopore on a silicon nitride membrane. As mentioned previously, CBD relies on the fact that when a high enough external voltage is applied, it drives a leakage current through the membrane and causes local defects, which allow the charge carriers to tunnel through and increase the leakage current.

Once the defects form a connected path, the sudden increase in voltage caused by the dielectric breakdown will blast through, and a nanopore is formed. This process usually leaves a relatively small pore with a diameter between 1 and 3 nm. In our automated process, because of the changing leakage current, a threshold of usually 25 nA is applied. When a breakdown occurs, the sudden increase of current will go over the threshold, and the software will register fabrication as completed, and the voltage will no longer be applied.

An I-V measurement is then conducted, where a cylindrical pore model is assumed to calculate the pore diameter[19]. Equation 1 for pore size is implemented in the software.

$$d = \frac{G}{2\sigma} \left(1 + \sqrt{1 + \frac{16\sigma t}{\pi G}} \right) \quad (1)$$

Here, d is the pore diameter, and G is the pore conductance, while σ is the solution conductivity, and t is the membrane thickness.

If no pore is detected, we can either repeat the fabrication process right away or go to conditioning, where if the pore does grow, then everything is good; and if the pore does not grow, the previous fabrication is likely a false positive, and we can repeat fabrication.

Once a pore is detected (defined by having a size larger than or equal to 1 nm under the I-V measurement), it moves on to conditioning, where it is placed under alternating small voltages to enlarge the pore to the size of choice. In preparation for the conditioning process, the electrolyte also needs to be changed. We now flush 600 μ L of 3.6 M LiCl at pH 8 (buffered with HEPES) into both sides of the flow cell. Although it is unclear at a fundamental level how nanopores grow under electrical stress, work published by our group has shown that the growth we observed is most likely due to electrochemical dissolution of the membrane material[20,21].

During conditioning, in order to monitor pore growth, quick measurements were done every 5 cycles, where each cycle consists of one period of positive voltage and one period of negative voltage. This quick I-V measurement only uses 2 points (± 0.2 V) to estimate the pore size. The automated software would initially choose $\frac{1}{3}$ of the breakdown voltage during fabrication, but users can adjust it however they see fit, depending on the growth rate. Once the quick measurements show that the pore has a size exceeding the target size, the conditioning process is stopped automatically. Figure 1.9 shows the complete automated fabrication and conditioning process.

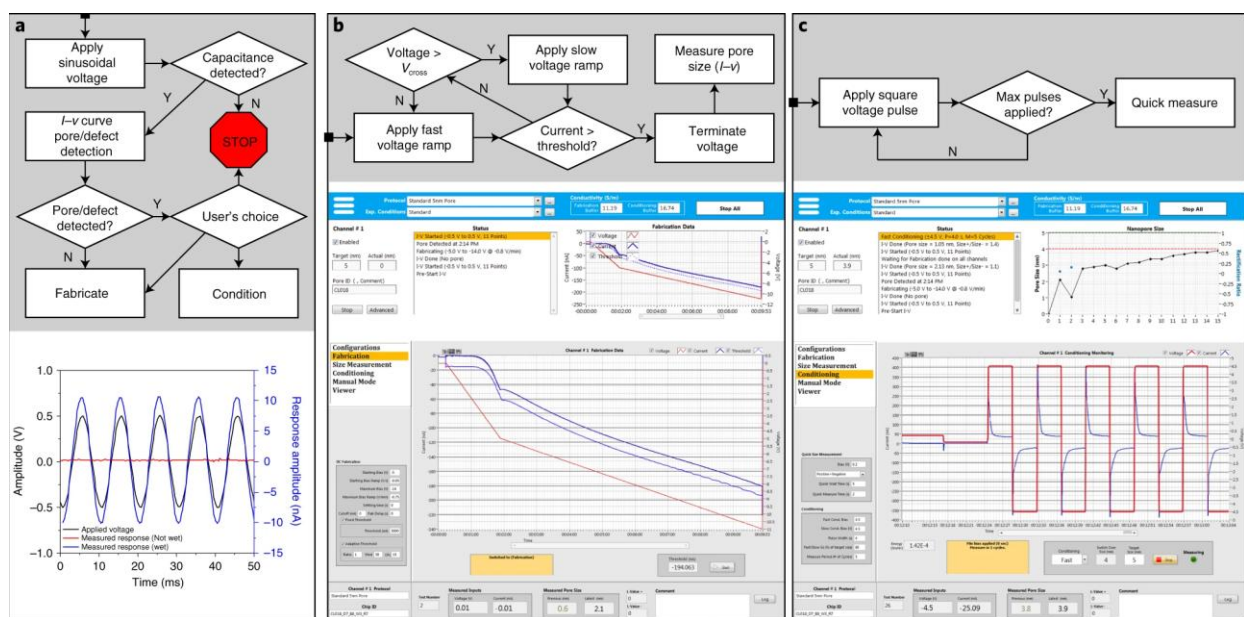


Figure 1.9 Fabrication and conditioning software interface, and the algorithm it used to decide how to proceed with the pore-making process. From *Solid-state nanopore fabrication by automated controlled breakdown*, Waugh et al., 2019. Copyright Nature Protocols 2019, reprinted with permission.

Finally, the pore is sent in for a complete I-V measurement to more confidently determine its size. If the size measured here is more than 0.5 nm smaller than the target size, the pore goes back to conditioning until the target size is reached. Figure 1.10 shows the I-V measurement.

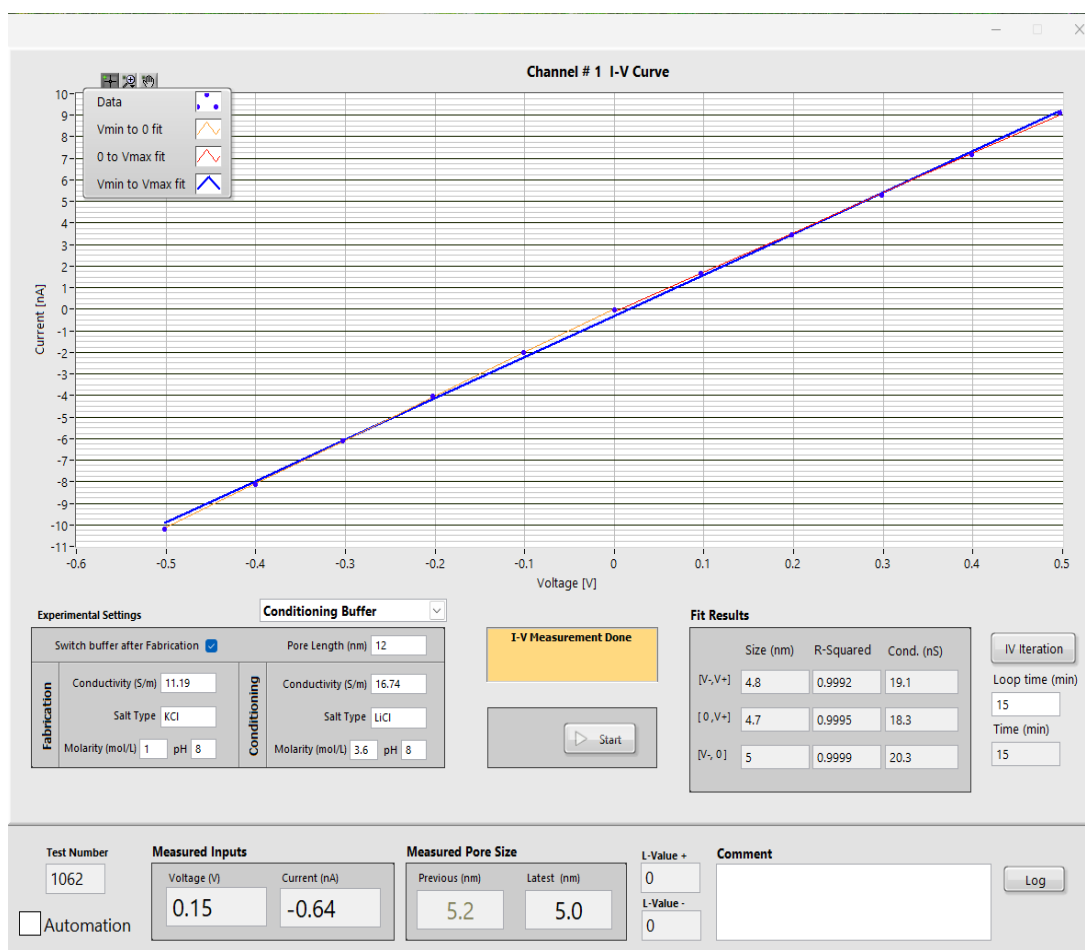


Figure 1.10 I-V measurement result of a ~5 nm pore.

After the I-V measurement shows the pore size is within 0.5 nm of the desired size, or any number larger than, the flow cell can be taken out, and the pore-making process is mostly complete. We then measure the 1/f noise of this nanopore, which will be discussed in detail in the next section, and if the noise is satisfactory, it can be used for experiments.

Otherwise, depending on the specific experiment and how strict it is in terms of pore size, if a bigger pore is acceptable, we can put the flow cell back into the instrument and further condition it, in the hope of better stability and lower noise. However, reconditioning does not always show success in lowering the noise. If the pore is too large or still too noisy for experiments, they have to be discarded.

1.3. Nanopore Sensing and Analysis

Our data acquisition is done using the Chimera VC 100, which has a sampling rate of 4.17 MHz and a full bandwidth of 1 MHz. To apply external voltages and measure the ionic current through the pores, we used a pair of Ag/AgCl electrodes to connect the VC 100 unit and the flow cell. The Chimera VC 100 head stage is located inside a Faraday cage, where it is connected to the flow cell housing the nanopore chip. This way, it can effectively shield the pA-level measurement process from outside mechanical and electrical noises. Figure 1.11 shows photographs of our lab setup. However, in experiments, because of the noise level at full bandwidth, we often have to apply a low-pass filter with a cutoff frequency of a few hundred kHz during analysis for a sufficient signal-to-noise ratio. The choice of cutoff frequency depends on the noise level of the pore and the signal depth generated by the analyte molecules.

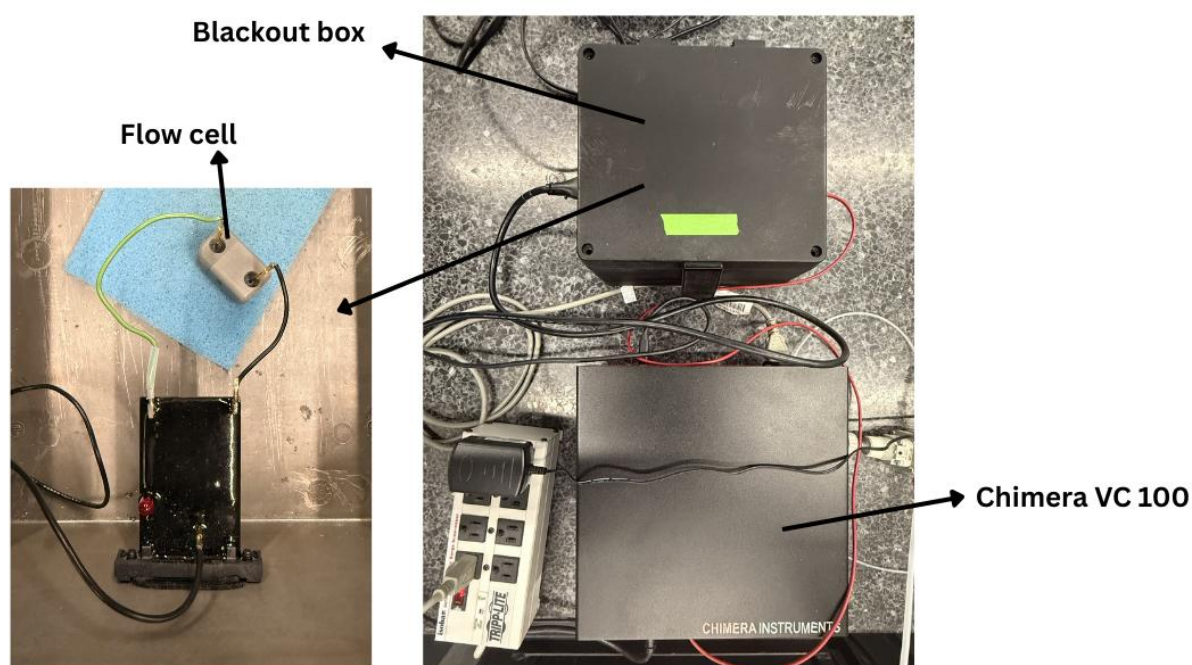


Figure 1.11 Set up of the sensing equipment, Chimera VC 100, and the Faraday cage, with a demonstration of how the flow cell is connected to the instrument.

Before we test if the pore is indeed functional (ie, will pass analyte molecules), we have to check if the noise is low enough for experiments. By connecting the flow cell to the Chimera, we sample for a short period of time (usually 10s of seconds to a couple of minutes, depending on the user). Then, we use software to plot the power spectral density to reveal the noise. Figure 1.12 shows the power spectral density and RMS noise plot of an adequate solid-state nanopore.

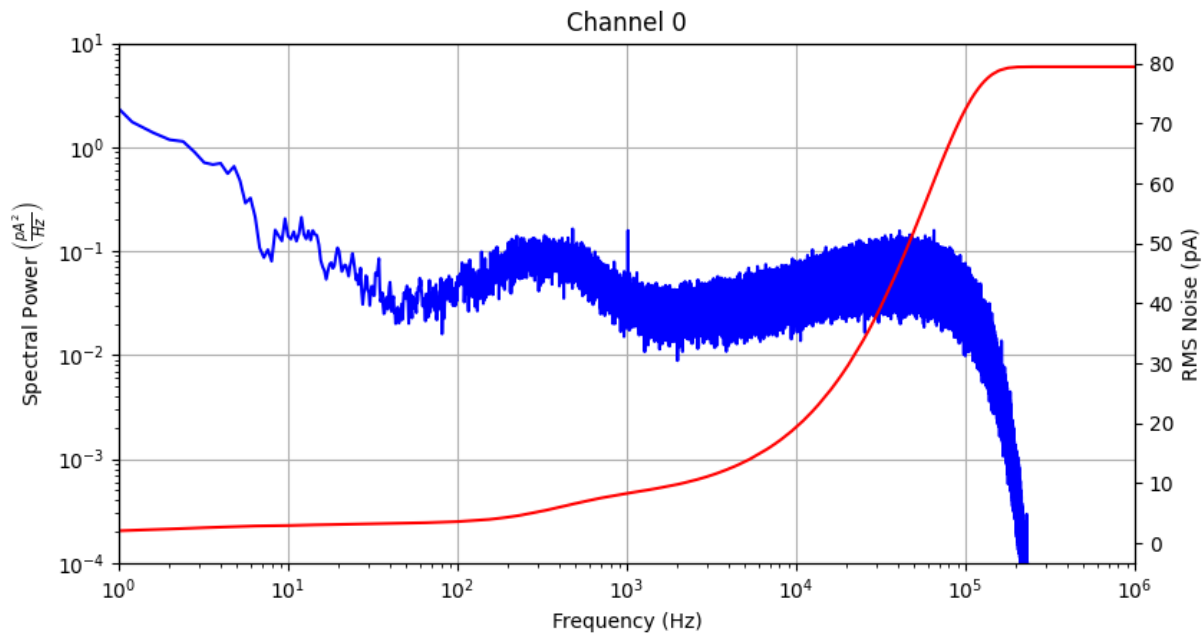


Figure 1.12 Power spectral density and RMS noise of a nanopore, using an 8-pole digital low-pass Bessel filter with a 200 kHz cutoff frequency.

If the noise level is adequate for experiments (usually we expect an RMS noise lower than 100 pA, and a spectral power of $\sim 10 \left(\frac{\text{pA}^2}{\text{Hz}} \right)$ at 1 Hz), we can then flush in analytes to test if they pass through the pore. When they do, these events will be shown as the current blockage. Figure 1.13 shows an example.

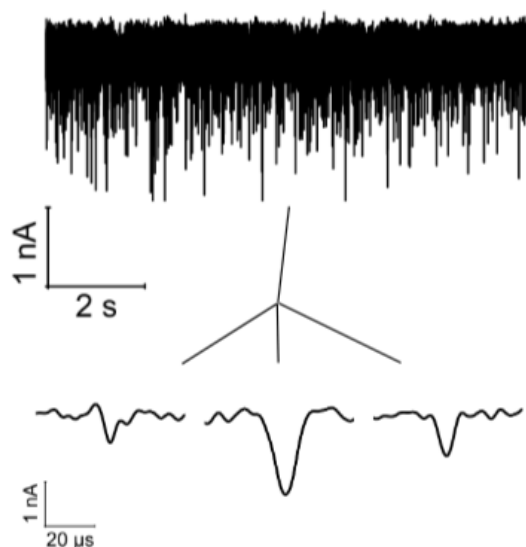


Figure 1.13 10-second current traces of heparin, 500 nM, in 4 M KCl at pH 4, sampled by Chimera VC 100; a 200 kHz low-pass filter was applied for sufficient SNR.

On rare occasions, it is possible for seemingly satisfactory nanopores to not pass analyte molecules, and no events are observed. It is most likely because there would be multiple small pores fabricated during dielectric breakdown, and the combined current is enough to be recognized as a bigger pore, but none can sufficiently act as a single-molecule detector.

To extract information needed in nanopore translocation events, there are two generations of data analysis software used in our lab, and they both operate on very similar processes and premises. Once the data collected is loaded into the software, we need to filter and process the data. The filtering degree and cutoff frequency depend on the user's choice to maintain SNR for different experiments and nanopores. Then we can find events. Here, a threshold (how much the signal deviates from the baseline) would be manually set, and the software would automatically detect these current blockages, recognize them as events, and store them in the database. The events found then have to be fitted, where event information is extracted. The fitted event information is stored in the database again. Many nanopore data

analysis software have adopted the CUSUM[22] algorithm to perform event finding and fitting. CUSUM allows adaptive event detection with respect to the baseline and the noise level, also fitting events at multiple levels, thus giving more accurate event information.

After the events are fitted, the information extracted can be used for further analysis. The software provides visualization tools to examine the data more directly. All information extracted from events can be plotted, and customized axes and plot types are also integrated for personalized use. Clustering and capture are available as well, and individual events can also be plotted for a closer, more detailed observation. Figure 1.14 demonstrates the process which Poriscope operates on.

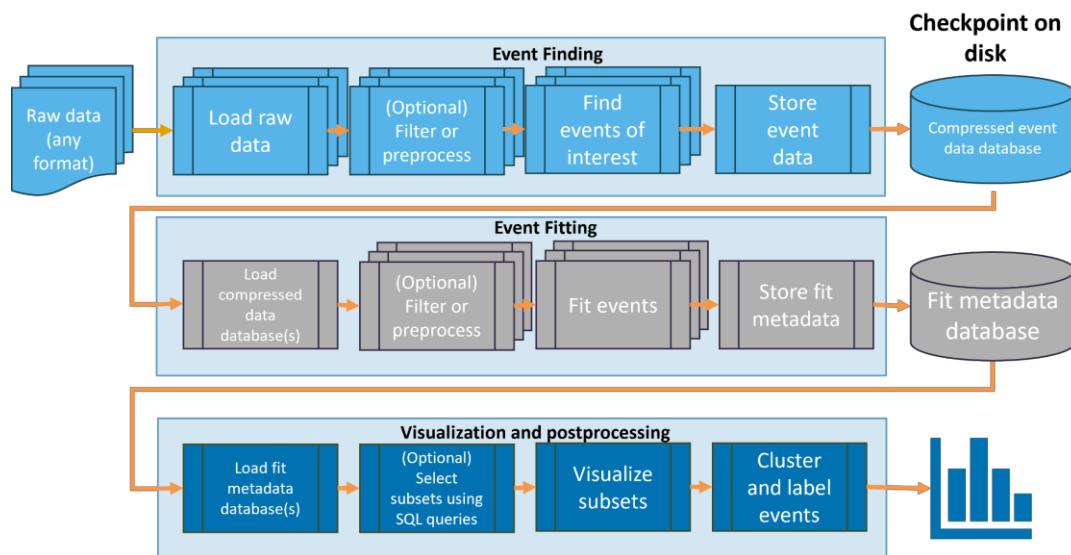


Figure 1.14 Steps followed in analyzing nanopore data with Poriscope. From *Poriscope: A Configurable Pipeline for Nanopore Analysis*, Gonzalez et al., 2026, licensed under CC Attribution 4.0.

Both generations of analysis tools used by our lab used CUSUM for event detection and fitting. Nanolyzer, the earlier model, developed by Northern Nanopore Instruments, has since been discontinued. In the absence of Nanolyzer, Poriscope[23] has been developed to replace it.

Poriscope and Nanolyzer work very similarly in terms of data processing, but one major improvement we made with Poriscope is that it allows plug-ins. Users can write their own programs that can be integrated into the existing Poriscope workflow, based on their specific needs to process and analyze the data. Poriscope gives more flexibility and extensibility and allows users to customize a workflow more suitable for their data.

1.4. Polysaccharides

Carbohydrates are sugar and starch molecules that serve as predominant nutrient sources in our daily life and are vastly useful across chemical, biological, and medical fields. Carbohydrates are composed of monosaccharide units, and polysaccharides refer to carbohydrate molecules composed of more than 10 monosaccharide units through glycosidic bonds[24]. They are generally long chains that can branch and form other complex structures, as Figure 1.15 schematically demonstrates, and their molecular weight can range from a few thousand to millions of Daltons[25].

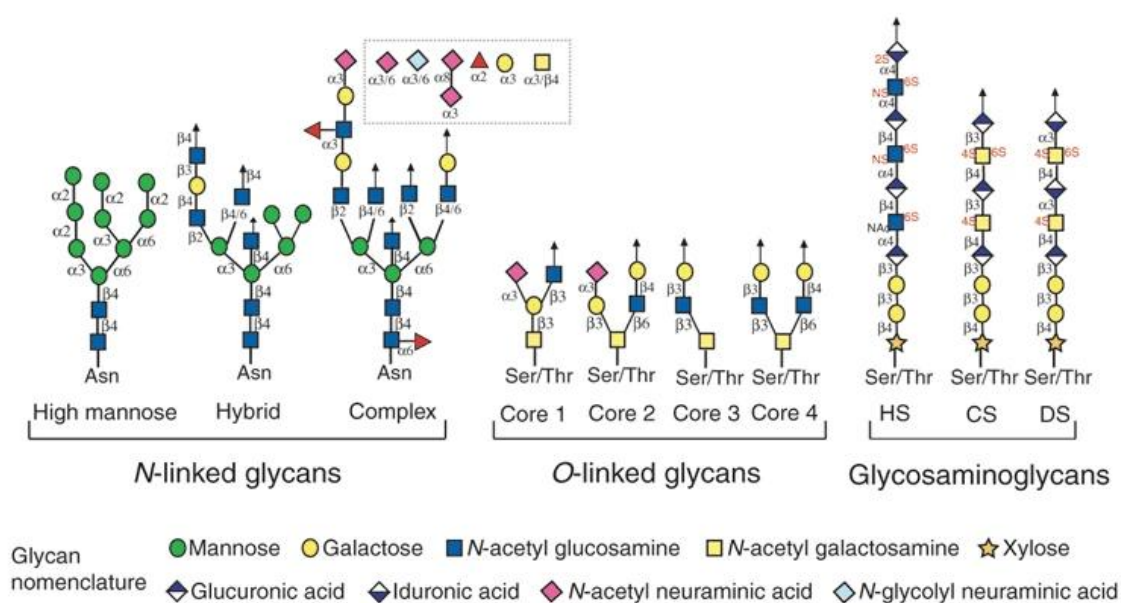


Figure 1.15 Examples of different structures of polysaccharides. From *Glycomics: an integrated systems approach to structure-function relationships of glycans*, Raman et al., 2005. Copyright Nature Methods 2005, reprinted with permission.

The building blocks of polysaccharides, or glycans, are monosaccharides[26]. These smaller units can have hundreds of different structures, and two different glycosidic linkages can also stem from a single monosaccharide, with two possible stereoisomers[27]. As they form oligosaccharides and polysaccharides, the structure becomes even more drastically

complex. Glycans are categorized into three main types: N-linked, O-linked, and Glycosaminoglycans (GAGs)[27]. N-linked glycans are the most common, where a group of sugar molecules are attached to a nitrogen atom from a protein residue, specifically asparagine[28]. Similarly, O-linked glycans refer to the sugar group attaching to an oxygen atom in a protein[29]. GAGs, different from N- and O-linked glycans, are individual long and negatively charged chains of repeating disaccharide units[30]. Their complexity and variety make them extremely difficult to analyze.

Previous studies have demonstrated that solid-state nanopores can be utilized as a single-molecular sensor for polysaccharides, such as heparin[31], hyaluronic acid[32], and xylan[14]. However, many results depend on obtaining monodisperse samples and other specific conditions, but in practice, the polydisperse nature of many glycans would leave these results irreproducible, thus making differentiation and identification extremely difficult. Direct sensing and sequencing of glycans remain a challenging task in the community. Sugar molecules can have vastly different sizes, charge states, and structures, so when passing through a solid-state nanopore, they could be too fast for state-of-the-art instruments to resolve or not charged enough for a full translocation. Therefore, we are still in need of a more efficient and cost-effective method for glycan sensing.

From the glycan family, GAGs are considered more ideal in terms of solid-state nanopore sensing because they are negatively charged, and the non-branching long-chain structure makes them easier to translocate through. Heparin is a GAG, composed of repeating disaccharide units with one iduronic acid and one glucosamine, as Figure 1.16 shows. The sulphate and hydroxyl groups on heparin can keep the molecules negatively charged in any protonation state. In solid-state nanopore studies, the kinetics of DNA are mostly well established; they translocate through the pore due to electrophoresis[33,34]. As heparin

molecules are also negatively charged, just like DNA, we suspect that their translocations would be of a similar nature.

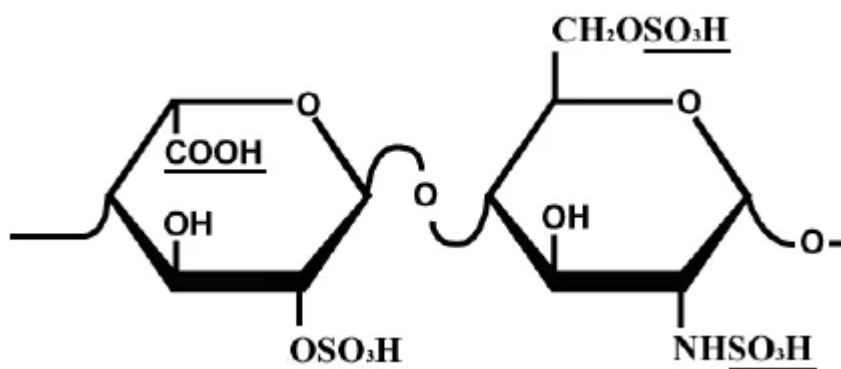


Figure 1.16 Molecular structure of one heparin disaccharide repeat. From *Heparin-Mimicking Polymer Modified Polyethersulfone Membranes – A Mini Review*, Xiang et al., 2014, licensed under CC Attribution.

From November 2007, incidents of allergic reactions after heparin treatment have been more frequently reported. These hypersensitivity reactions can cause hypertension and, in extreme cases, lead to death[35]. After recalling all lots of the contaminated heparin, oversulphated chondroitin sulphate (OSCS) has been identified as the major contaminant and could take up as much as 30% of the heparin product[35]. OSCS is a variation of the naturally occurring polysaccharide chondroitin sulphate (CS), whose molecular structure is shown in Figure 1.17, but OSCS has a higher degree of sulphation. Here, the R's represent possible attachment sites for sulphate groups. Naturally occurring CS only have two possible configurations, chondroitin-4-sulphate and chondroitin-6-sulphate, so only R_1 or R_2 has a sulphate group attached, and the rest have a simple hydrogen atom. CS therefore only has one sulphate group. For OSCS, all four sites have sulphate groups, thus making it four times as sulphated as CS.

Although CS is generally considered safe for humans and can be used as a treatment for osteoarthritis, OSCS-contaminated heparin can cause serious consequences. While OSCS and heparin have similar molecular structures, both being long chains of disaccharide repeats derived from pyranose rings, OSCS does not have anticoagulant properties.

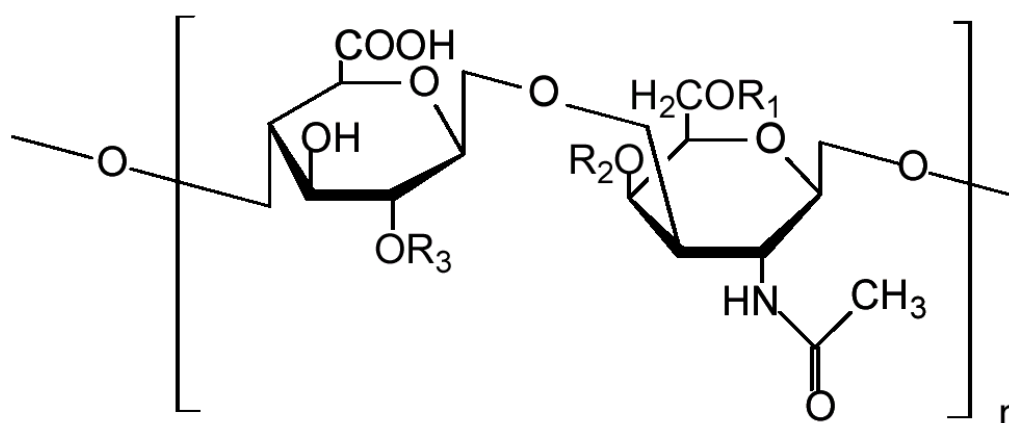


Figure 1.17 Molecular structures of chondroitin sulphate. From *Nutraceuticals*, Chapter 14, Gupta et al., 2021. Copyright Elsevier Books 2021, reprinted with permission.

Therefore, we study heparin with solid-state nanopores because of its practical use in medical treatments, the challenges we face in analyzing small sugar molecules, and to gain more insights into their characteristics and provide more possibilities to differentiate the molecules in future studies.

Many attempts to differentiate OSCS and heparin have been made in the community. HPLC[36], NMR[37], CE[38], Py-MS[39], IR[40], fluorescence assays[41], and solid-state nanopores[31] have all been explored. Figure 1.18 shows traces of heparin and chondroitin sulphate translocations through a solid-state nanopore. Characteristics and features to distinguish the two molecules are hard to discern from these traces. Because of their similar chemical structure, charge state, and molecular weight, we have yet to find a reliable and replicable method for commercial quality assurance. Many traditional macromolecular

analyses also face challenges in processing time, cost, and sample requirements. A lot of instruments require samples of a certain purity and polydispersity index that are extremely expensive to obtain for commercial use.

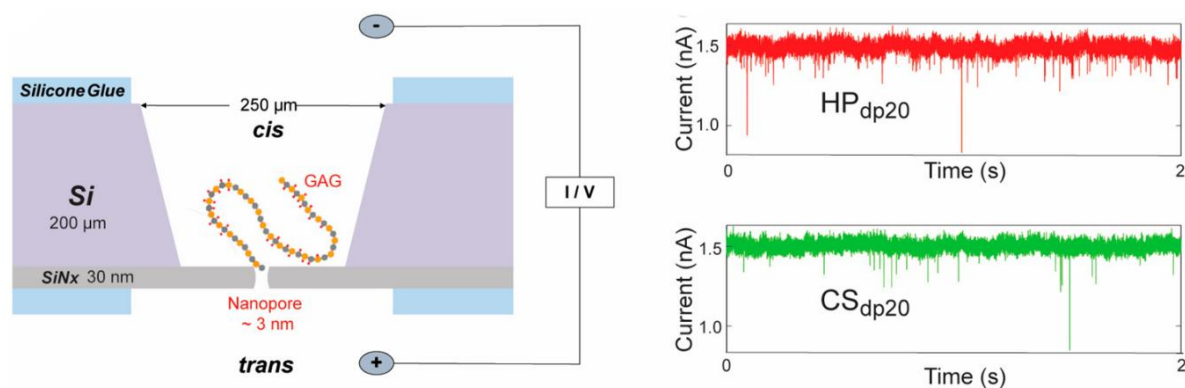


Figure 1.18 Illustrative setup of a solid-state nanopore system and representative trace of sugar molecules. The setup to sense a GAG molecule (left); representative traces of heparin and OSCS through a solid-state nanopore (right). From *Single Molecule Identification and Quantification of Glycosaminoglycans Using Solid-State Nanopores*, Im et al., 2019, Copyright ACS Nano 2019, reprinted with permission.

1.5. Literature Review

Xylan is a polysaccharide found in plant cell walls, and it is the most abundant polysaccharide after cellulose[42]. Xylan carries acetyl groups in the backbone to properly provide support to cell walls, but both chemical modification and naturally occurring mutations can cause deacetylation that could potentially compromise cell wall integrity[43]. As solid-state nanopores are on the rise as the next generation of single-molecular sensors, Cai et al.[14] conducted a study detecting and discriminating xylan molecules.

From their experimental results, xylan successfully translocated through a ~ 4 nm solid-state nanopore, and typical signals that range from 5 to 20% of the open current were observed. Xylan extracted from brittle leaf sheath1 (bs1) exhibits a hyperacetylation mutation. By comparing mutated xylan to properly acetylated xylan (extracted from wild-type Nipponbare, NP), it is shown that bs1 xylan has a shallower blockage, and the difference is very much visible to distinguish the two.

To further strengthen the argument, another experiment was done to compare normal xylan and deacetylated xylan. Although NP xylan and the deacetylated counterparts demonstrated no significant differences, the bs1 xylan and the deacetylated bs1 xylan populations were clearly separated, with the bs1 population having a shallower blockage. This helped the authors to once again confirm that xylan's acetylation level is inversely related to its signal depth.

The solid-state nanopore system was also tested to differentiate different types of xylan. Arabinoxylan (AX) and glucuronoxylan (GX) have slight variations in terms of chemical structures that result in two different types of plant walls. When examining AX and GX xylan, it is observed that AX xylan, which is what the study has been using for previous experiments, has signals no deeper than 20% of the open current; GX xylan has a much wider distribution, as the event's depth can be as low as 5% and as deep as 40%. This major difference can help

us quickly identify which type of xylan is on hand. Figure 1.19 summarizes the main findings of this study.

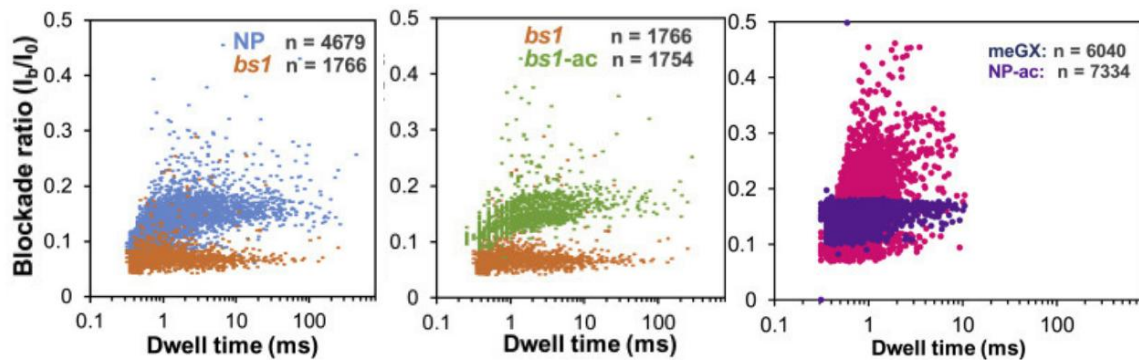


Figure 1.19 Scatter plots of dwell time versus blockage ratio of different plant polysaccharides. Plots demonstrated normally acetylated xylan (NP) and hyperacetylated xylan (bs1) having visually different populations, with NP exhibiting a deeper blockage (left); hyperacetylated xylan (bs1) and deacetylated xylan (bs1-ac) confirm the pattern with bs1 having a deeper blockage (middle); meGX has a wider distribution of blockage depth, differentiating it from the deacetylated NP population (right). From *A solid-state nanopore-based single-molecule approach for label-free characterization of plant polysaccharides*, Cai et al., 2021, licensed under CC BY-NC-ND 4.0.

Cai et al. successfully demonstrated that solid-state nanopore systems have the ability to sense complex polysaccharides like xylan and have made discriminating between different types and acetylation levels of xylan possible.

Hyaluronic acid (HA) is a polysaccharide that has taken an important role in medical and commercial usage. Because of its ability to act as a lubricant in the skin, joints, and eyes[44], HA has been popularized in the medical and beauty industry to treat osteoarthritis, dry eye, wrinkles, and act as a filler in cosmetic procedures[45]. Not only do they have great potential as marketable goods, but HA can also be important biomarkers in pathology, especially due to its relative abundance and molecular weight distribution[46]. While gel migration and other methods have been effective in determining size distribution, Rivas et al.[32] demonstrated that solid-state nanopores can also be used for these purposes.

In nanopore analysis, the equivalent charge deficit, or ECD, represents the amount of charge blocked by the molecule during translocation. It is a metric to more accurately sort biomolecules by size than using passage time or blockage depth alone, by correcting for molecular folding, for example. It is defined as the current blockage multiplied by the dwell time:

$$ECD = \int I(t)dt \quad (2)$$

By translating the event signals into ECD and plotting the distributions, they have found that the ECD distribution contains very similar information to the gel method. Experiments have confirmed the viability of the solid-state nanopore systems for polydisperse, quasi-monodisperse HA, and HA extracted from synovial fluid, all showing that adequate molecular size distribution can be obtained from the ECD distribution of nanopore events. Figure 1.20 compares the gel migration and ECD extracted from solid-state nanopore systems.

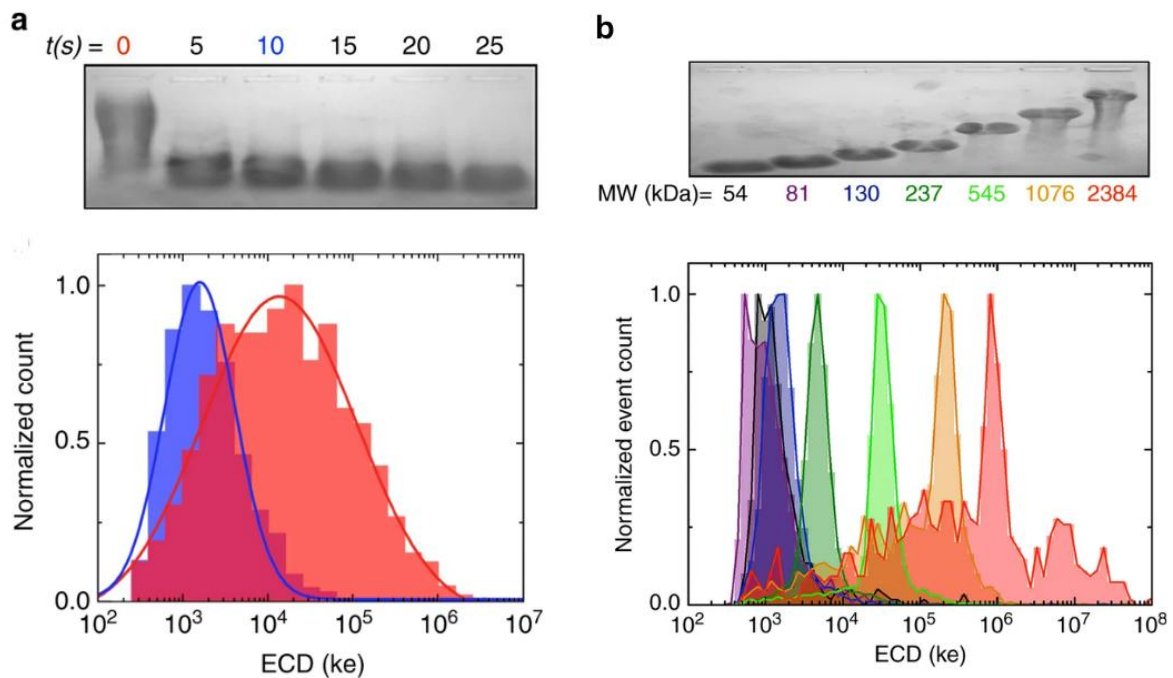


Figure 1.20 Comparison of gel migration and nanopore ECD results. a) gel migration and ECD from the same sample of polydisperse HA. **b)** gel migration and ECD comparison from the same sample of quasi-monodisperse HA. Both show very similar results. *From Label-free analysis of physiological hyaluronan size distribution with a solid-state nanopore sensor, Rivas et al., 2018, licensed under CC BY 4.0.*

Their study has shown a new possibility for solid-state nanopores in single-molecule analysis, other than identification or quantification. The gel migration takes hours, even up to days, to obtain results, but these distributions extracted from nanopore systems can be constructed from hundreds to thousands of events, which would only take minutes.

Alginate is another polysaccharide derived from seaweed, and in the food industry, it has been popularized for its thickening properties[47]. It is also used as a reflux suppressant in medical practices. Karawdeniya et al.[31] have utilized solid-state nanopores to sense alginate. Various experimental conditions were tested, showing that the optimized sensing condition would be with a smaller pore size, ~ 5 nm in diameter and in a solution with higher conductivity, as the detection rate is 10x in 1 M KCl compared to 0.1 M KCl, and that a ~ 5 nm pore also increased the capture rate and provided a higher blockage ratio, as shown in Figure 1.21.

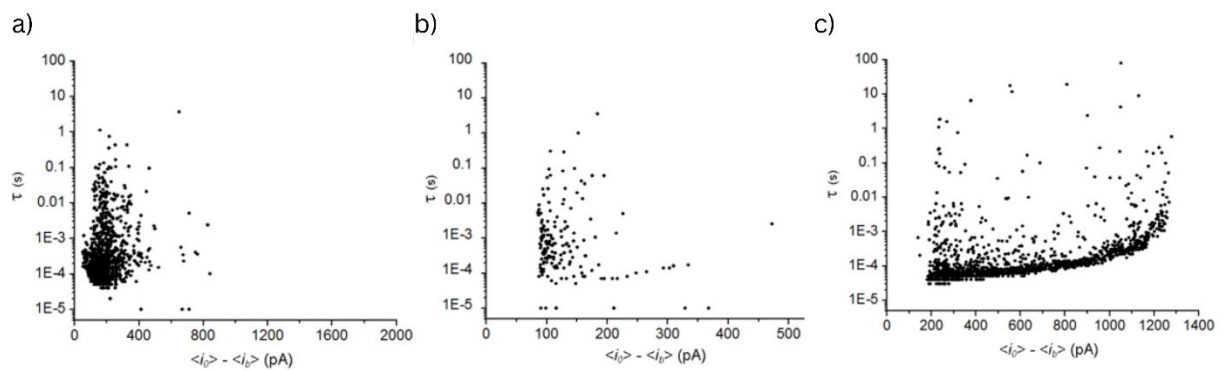


Figure 1.21 Comparison of alginate events in different salt concentrations and pore sizes. **a)** scatterplot of alginate events in 1 M KCl, pH 7, in a ~ 19 nm nanopore (~ 0.3 events/s), **b)** alginate events in 0.1 M KCl, pH 7, in a ~ 18 nm pore (~ 0.05 events/s). **c)** alginate events in 1 M KCl, pH 7, in a ~ 5 nm pore (~ 0.4 events/s). All analytes are in a concentration of 0.2% (w/v). A low-pass filter of 10 kHz was applied. From *Surveying silicon nitride nanopores for glycomics and heparin quality assurance*, Karawdeniya et al., 2018, licensed under CC BY 4.0.

A pH sweep was also done to examine the kinetics of alginate molecules, revealing that at more acidic environments where electrophoresis and electroosmosis enhance each other, alginate detection was maximized, as shown in Figure 1.22.

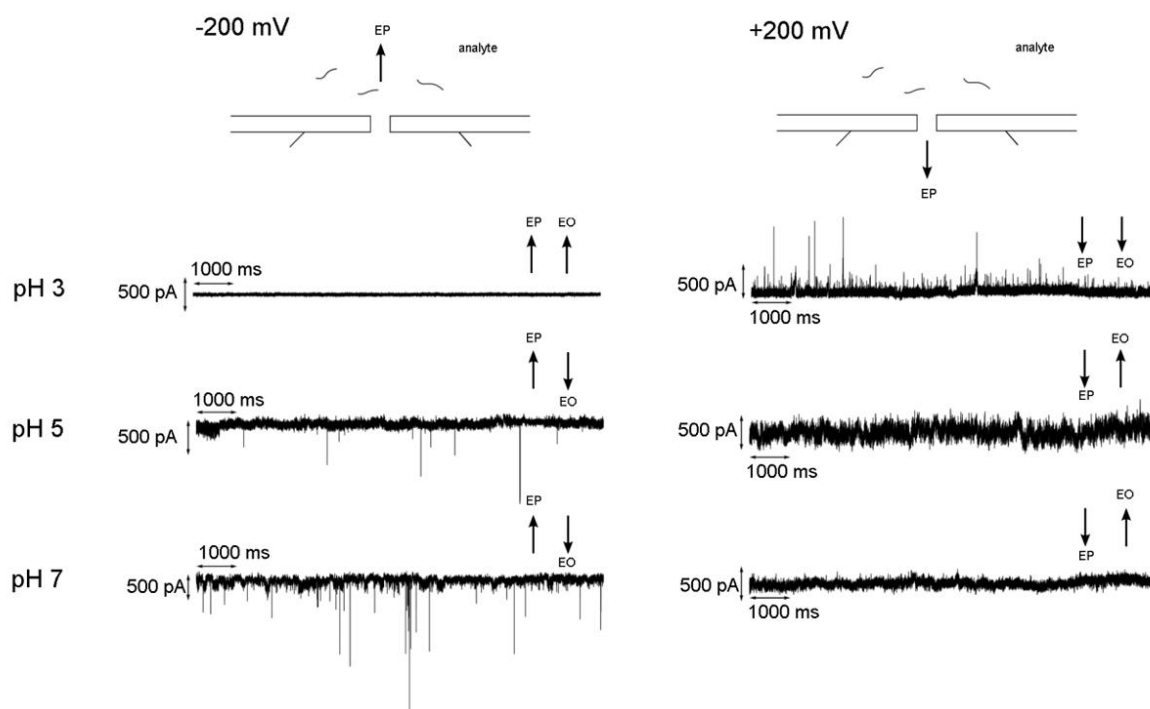


Figure 1.22 Alginate traces in different voltage polarities (± 200 mV) at pH 3, 5, 7, showing different translocation kinetics at different protonation states. From *Surveying silicon nitride nanopores for glycomics and heparin quality assurance*, Karawdeniya et al., 2018, licensed under CC BY 4.0.

In the same publication, Karawdeniya et al. also attempted to differentiate heparin and its contaminant OSCS with solid-state nanopore systems. Their data have shown that in 1 M KCl at pH 7, although heparin and OSCS both translocate on a timescale between 10 μ s and about 0.1 s, OSCS displays a population with a much deeper tail, which heparin signals do not have. A mixture of heparin and OSCS samples also shows a tail that is less prominent but present. Based on these results, they have demonstrated that a possible heparin quality control process can be achieved using a solid-state nanopore, as demonstrated in Figure 1.23.

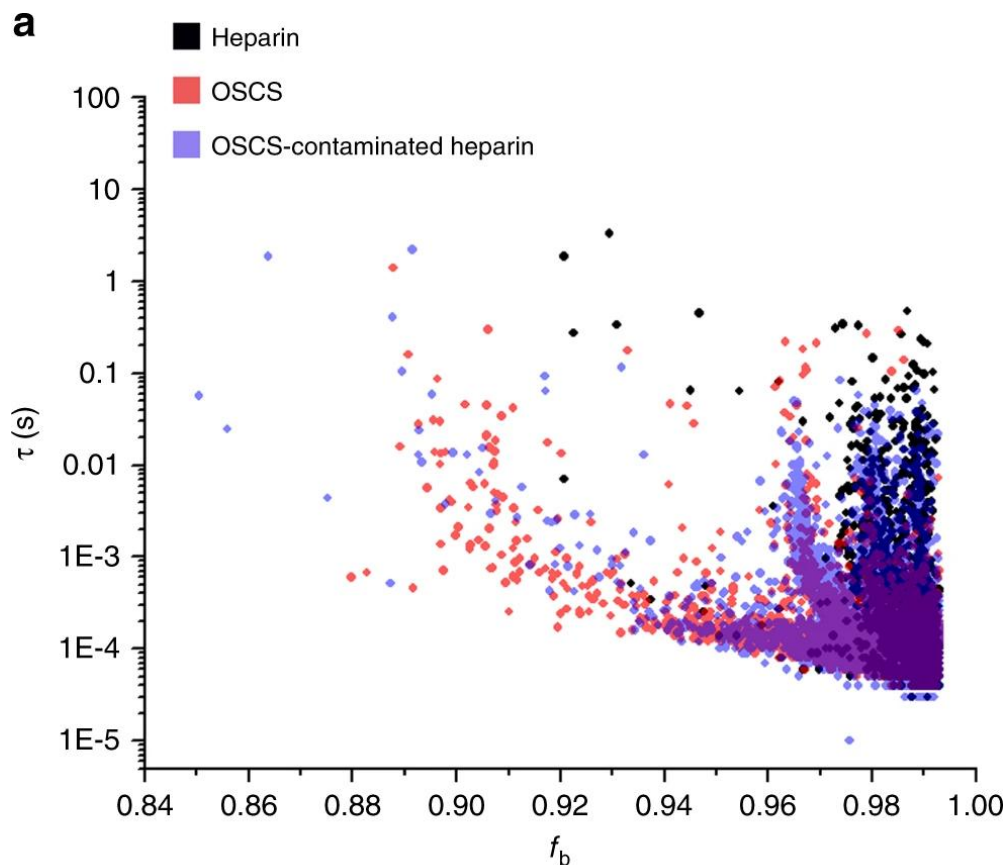


Figure 1.23 Populations of heparin, OSCS, and OSCS-contaminated heparin events, showing a wider distribution of blockage depth in OSCS (red) and OSCS-contaminated populations (purple), compared to the heparin population (black), making them visually identifiable. From *Surveying silicon nitride nanopores for glycomics and heparin quality assurance*, Karawdeniya et al., 2018, licensed under CC BY 4.0.

However, this unique characteristic of the OSCS population was not found to be replicable in other publications. Im et al.[15] have conducted a similar study aiming to separate heparin and chondroitin sulphate. Heparin and CS were prepared in 0.4 M KCl at pH 7.4, at a concentration of $\sim 1 \mu\text{M}$, and the two populations had no distinct feature that could be observed visually, as Figure 1.6 revealed. A cut-off frequency of 100 kHz was applied for their analysis.

Im et al. then trained a supervised machine learning algorithm to identify the analytes. Low molecular weight heparin and unfractionated heparin were also experimented with solid-state nanopore systems, and the data were used for machine learning. This method was welcomed with some success, as the identification success rate between any of the two types of heparins was above 80%, except between LMWH and CS. However, when identifying in a

pool of four, the accuracy dropped significantly to below 70%. Nonetheless, Machine Learning approaches show promise in identifying and quantifying different types of heparin molecules with solid-state nanopore systems.

entry	entity	training accuracy (%)	identification accuracy ^a (%)				average
			UFH	CS _{dp20}	Enox	HP _{dp20}	
1	UFH vs CS _{dp20}	100	94.6 ± 1.2	91.0 ± 1.5			92.5 ± 1.0
2	UFH vs Enox	100	95.9 ± 0.9		92.9 ± 0.9		94.4 ± 0.6
3	UFH vs HP _{dp20}	100	87.2 ± 1.6			82.1 ± 1.4	84.7 ± 1.1
4	Enox vs CS _{dp20}	100		73.8 ± 2.7	72.1 ± 1.7		73.0 ± 1.6
5	Enox vs HP _{dp20}	100			88.4 ± 2.3	86.4 ± 1.3	87.4 ± 1.3
6	HP _{dp20} vs CS _{dp20}	100		88.7 ± 1.8		83.3 ± 1.4	86.0 ± 1.1
7	pool of four	100	80.6 ± 1.6	65.1 ± 2.6	65.2 ± 3.3	64.8 ± 3.3	68.9 ± 1.4

Table 1 Accuracy of SVM (Support Vector Machine) identification with different molecules. From *Single Molecule Identification and Quantification of Glycosaminoglycans Using Solid-State Nanopores*, Im et al., 2019, Copyright ACS Nano 2019, reprinted with permission.

However, in practical cases, the difference between CS and OSCS could throw a dent in the identification accuracy, since the contamination percentage in heparin could vary and only goes up to 30%.

The ML algorithm was also used for identifying different types of heparan sulphate. Xia et al.[16] used solid-state nanopore systems to attempt to differentiate four types of synthetic GAGs in Figure 1.24.

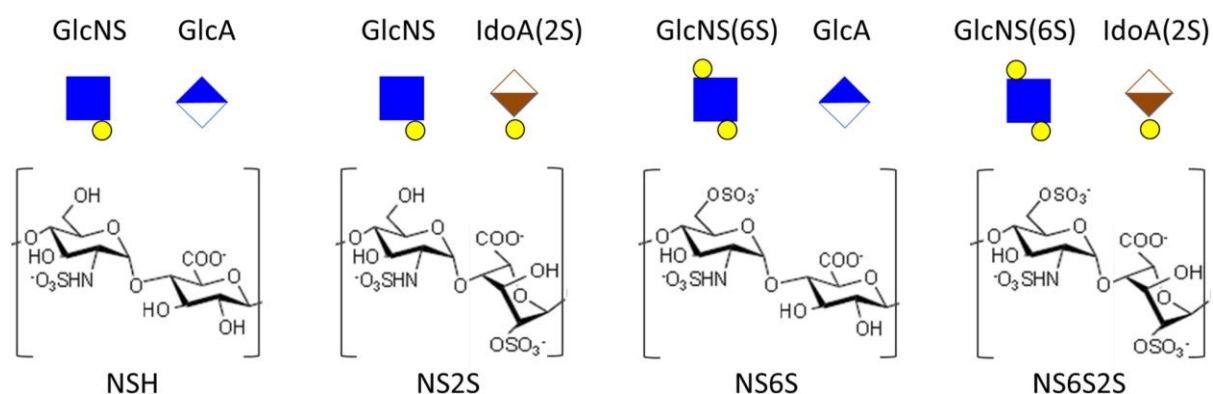


Figure 1.24 Molecular structures of the glycans used for machine learning, to identify with a solid-state nanopore system. From *Synthetic heparan sulfate standards and machine learning facilitate the development of solid-state nanopore analysis*, Xia et al., 2020, licensed under the PNAS license.

After training, the algorithm was able to identify different types of heparan sulphate with an average accuracy of 98.3%. The group also experimented with other machine learning models, and the results have shown that the Support Vector Machine (SVM) has the highest precision in Figure 1.25.

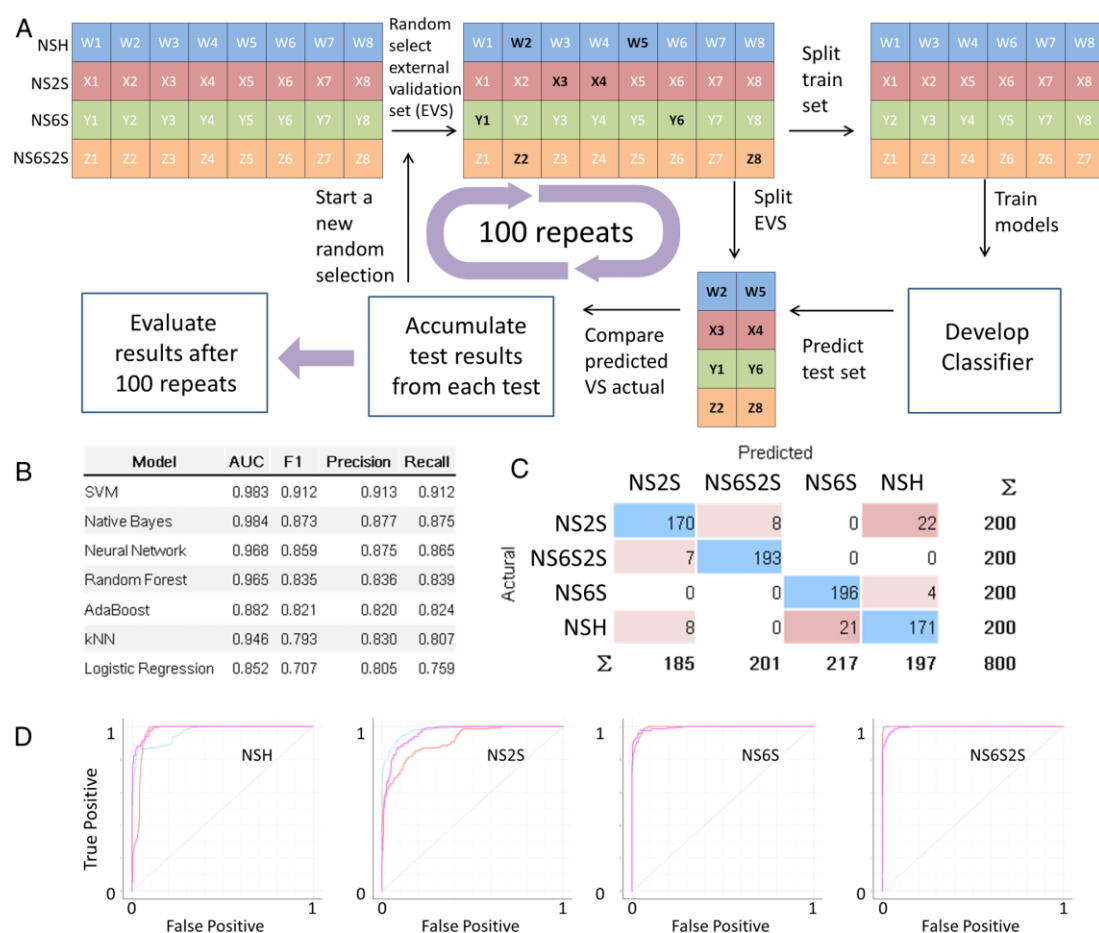


Figure 1.25 Machine learning process and results for the identification of glycans with a solid-state nanopore system. **a)** Training process of the algorithm to achieve identification, **b)** identification accuracy between different algorithms, showing SVM has the best precision overall, **c)** The confusion table of the SVM identifications, **d)** ROC curve for each molecule with the top three models (SVM: magenta, native Bayes: blue, neural network: orange). From *Synthetic heparan sulfate standards and machine learning facilitate the development of solid-state nanopore analysis*, Xia et al., 2020, licensed under the PNAS license.

Although these were exciting results and show promise of solid-state nanopore as a single-molecule sensor for polysaccharides, many challenges remain. As we see in previously discussed papers, in order to retain a sufficient SNR for analysis with state-of-the-art instruments, the data were often heavily filtered, keeping only slower events for analysis, and the majority of events were overlooked. The information obtained from these experiments was thus very limited, and the event signatures were heavily attenuated. Techniques that have been

explored to slow down translocations and improve resolutions include tagging, fragmentation, and nanopore modifications.

When the target molecules are too small or fast to properly resolve, tagging them with a binding agent could increase event depth and slow down the molecules. Heparin remains a very interesting molecule in the field, and its ability to bind with different proteins opens up many possibilities. Thyashan et al.[48] have demonstrated that heparin binds with FGF-1 proteins through a solid-state nanopore. As studies have previously shown and confirmed in this Thesis, heparin has a very fast and shallow population. Although FGF-1 has a much broader distribution in terms of event depth, the majority of the population is even shallower than heparin. However, their mixture exhibited a drastic shift. The heparin-FGF-1-bound molecules translocate through the pore as a stable unit, as the blockage depth exceeds the combined value of these two molecules alone, as shown in Figure 1.26. The bound population, although it has a wider distribution of timescales, has many events that remain very fast, at under 100 μ s.

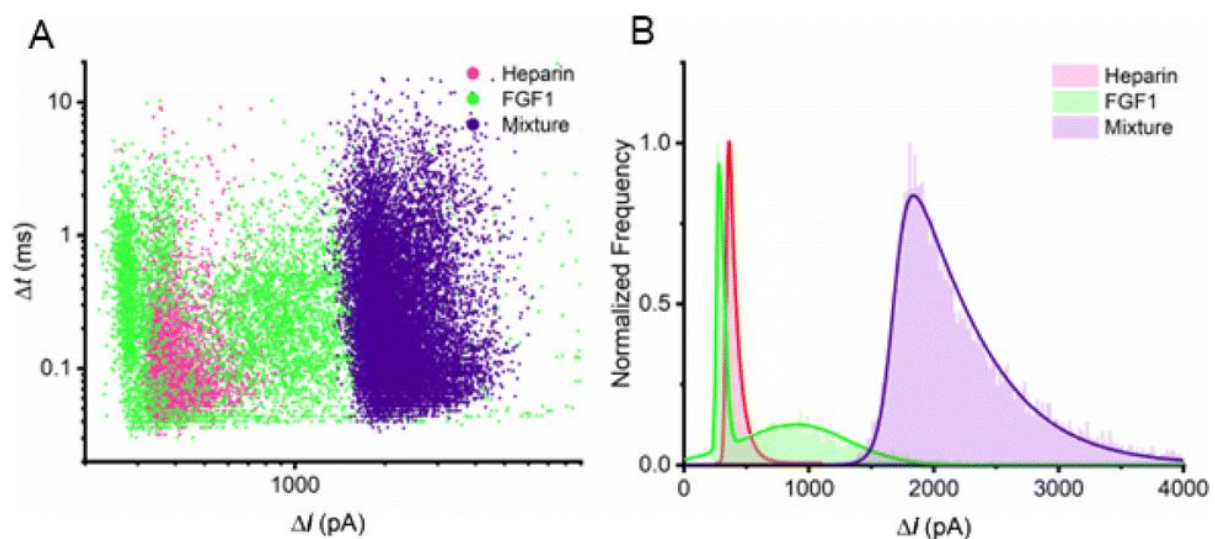


Figure 1.26 Scatter plot and histograms showing blockage depth of heparin, FGF1 and their mixture. a) Populations of heparin, FGF-1 protein, and their mixture, showing the mixture has a much deeper blockage depth, b) histogram of the blockage depth in a). From *Exploring single-molecule interactions: heparin and FGF-1 proteins through solid-state nanopores*, Thyashan et al., 2024. Copyright Nanoscale 2024, reprinted with permission.

While tagging provides a promising attempt that we could explore with future glycan studies by combining the molecules to create a more prominent signal that is easier to analyze, fragmentation provides insights from the opposite direction. Fragmentation works on the principle that we slice polysaccharides and oligosaccharides into smaller branches or monosaccharides, whose signals are known and well-studied. Then, through a computational process, we piece the entire molecular structure together. This fragmentation process is demonstrated in Figure 1.27.

Fragmentation of glycan molecules has seen success with protein nanopores, as Xia et al.[49] and Xiao et al.[50].

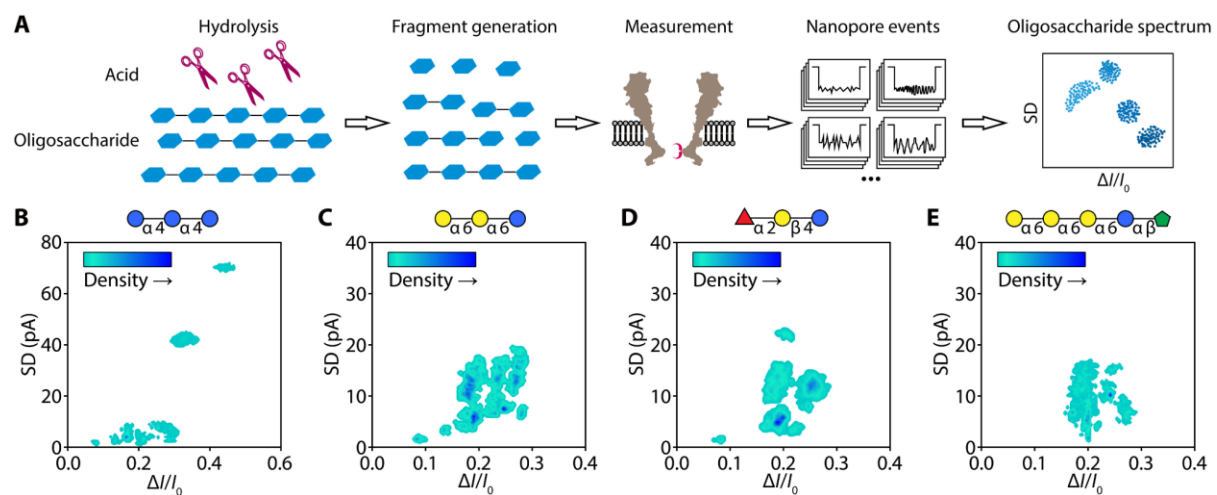


Figure 1.27 Demonstration and experimental results of the fragmentation method. a) Demonstration of the fragmentation method, b) representative populations of different oligosaccharides. From *Nanopore profiling and structure determination of oligosaccharides by fragmentation*, Xiao et al., 2026, licensed under CC BY-NC 4.0.

From direct sensing of fragments of poly- and oligosaccharides, both groups were able to reconstruct the complete molecular structure with a high success rate (over 90%). In these cases, protein nanopores were chosen because of their reproducibility and small diameter compared to solid-state nanopores. However, glass nanopipettes (a type of solid-state nanopore, made by pulling glass capillaries) filled with a carbon nitride film have also demonstrated similar capabilities. Synthesized carbon nitride can create nanopore openings as

small as sub-one-nanometer. Guo et al.[51] have shown that CN nanopores can successfully sense and discriminate monosaccharide, disaccharide and trisaccharide molecules with high resolution, as shown in Figure 1.28.

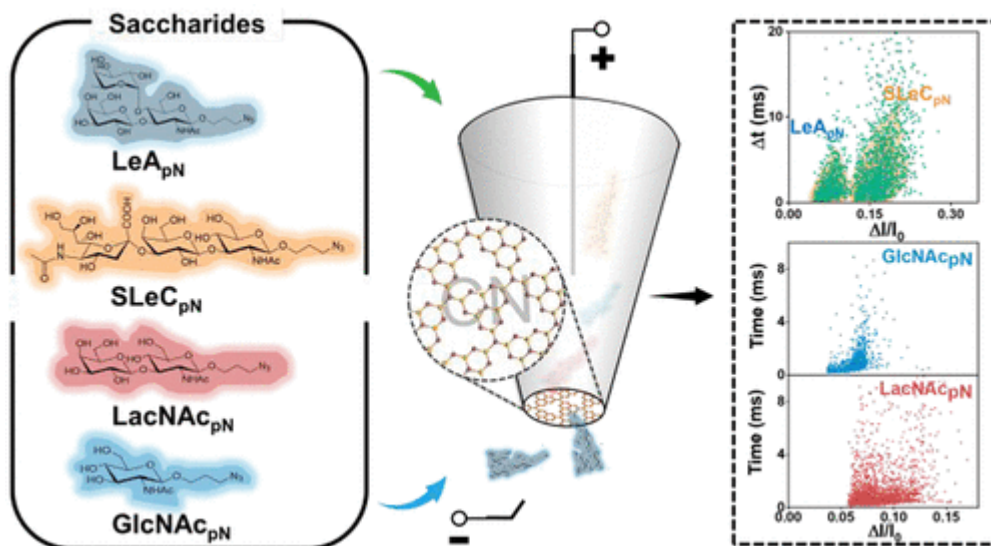


Figure 1.28 Molecular structures (left) of the saccharides experimented with the CN nanopore (middle) and their populations, exhibiting visual differences. From *Single-Molecule Discrimination of Saccharides Using Carbon Nitride Nanopores*, Guo et al., 2024. Copyright Nano Letters 2024, reprinted with permission.

Another route that could be explored is pore modification/functionalization. We have yet to see modified solid-state nanopores specifically targeted for glycan sensing, but previous studies have proven the feasibility of this route for other analytes. For example, Awasthi et al.[52] have developed a protocol (Figure 1.29) to attach different anti-fouling materials onto the surface to minimize pore-molecule interactions.

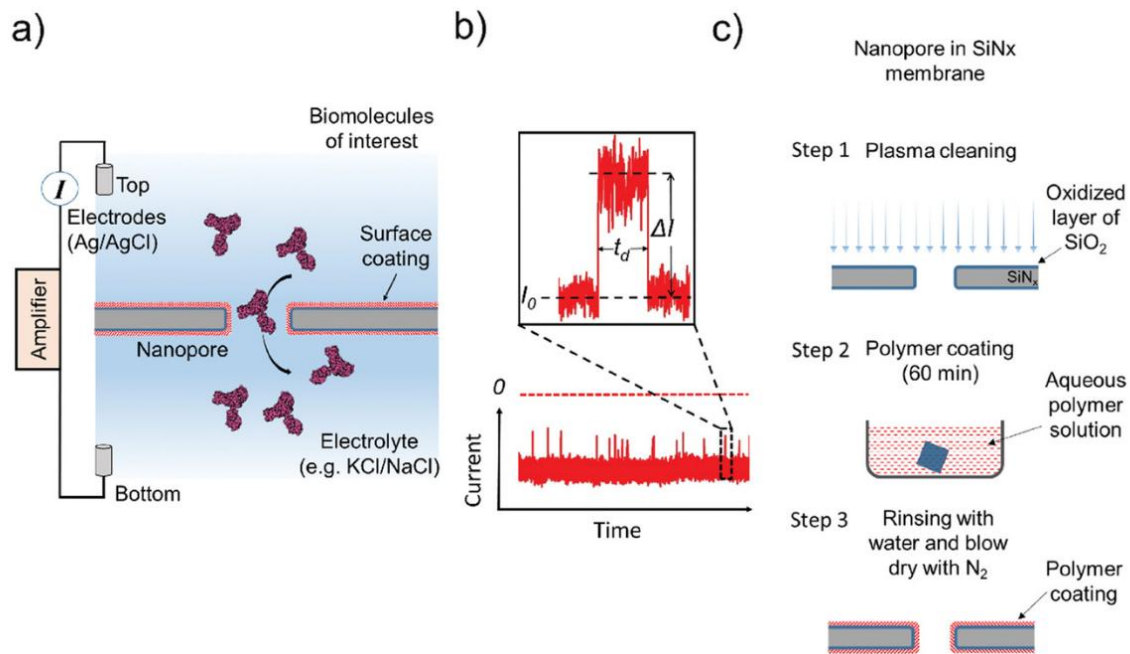


Figure 1.29 Schematics of a functionalized pore, its representative traces, and the process of surface coating. a) schematic of analyte molecules passing through a surface-coated solid-state nanopore, b) representative trace and event of the translocation, c) demonstration of the coating process. From *Polymer Coatings to Minimize Protein Adsorption in Solid-State Nanopores*, Awasthi et al., 2020, licensed under CC BY 4.0.

Their coatings have proven useful to sustain a longer use time of the pore and more accurate volume estimation of the target molecule. Tanimoto et al.[53] have similar ideas of nanopore surface coating, but they achieved this through chemical grafting. The PEG chains are immobilized on the solid-state nanopore surface, and this functionalization allows them to target the detection of selective proteins of interest. These studies on nanopore functionalization could bring enlightenment to future glycan sensing, as we have yet to explore the complex and robust chemical properties they possess and their interactions with various polymers.

2. Chapter 2. Dynamics of Heparin Translocations through Solid-State Nanopores

Kexin Zhao, Matthew Waugh, Kyle Briggs, Mohammad P. Jamshidi, Janelle Sauvageau, Vincent Tabard-Cossa

Abstract

Carbohydrates are one of the many biomolecular building blocks of life, with many practical applications in medicine. However, current methods for analysing these biomolecules struggle to operate at the single-molecule level. Carbohydrate sensing with solid-state nanopores has recently been explored but has proven difficult due to their structural complexity and the rapid translocation through the pores in the case of short polysaccharide chains. Among the numerous forms they can take, the best-known example is heparin, a widely used anticoagulant that has a closely related but toxic contaminant. In this work, we study the kinetics of capture and translocation of heparin molecules passing through a solid-state nanopore based on experimental results and signal simulations, seeking to understand the dynamics of passage. We examine the molecular interaction with the pore as a function of pH to elucidate the conformational dynamics of translocation. Despite using state-of-the-art electronics, we find that the durations of translocation through bare silicon nitride pores are too short to provide the resolution needed to reliably distinguish heparin from its contaminants. En route, we shed light on the physical mechanisms that govern translocation and suggest practical means by which future work might overcome these limitations.

2.1. Introduction

Carbohydrates are biomolecules crucial to life[54], but their complexity makes them difficult to analyse. Not only do hundreds of structurally distinct monosaccharides exist, but

each monosaccharide can form glycosidic bonds with different regiochemistries and two possible anomeric configurations (α and β).^[54,55] Nuclear Magnetic Resonance (NMR) is often used to determine a monosaccharide's identity and linkages^[54,55], but for large bacterial carbohydrates, hours of NMR spectral recording lead to results requiring careful and lengthy interpretation. Size Exclusion Chromatography (SEC) can provide insight into a large carbohydrate's size but also requires long chromatography runs. As a result, carbohydrates are poorly understood compared to their simpler protein, lipid and nucleotide counterparts. To speed up analysis, strategies such as lectin microarrays^[56] can provide an overview of carbohydrates in cells or tissues. However, such equipment is not widely accessible and is highly dependent on having access to a range of purified lectins for various carbohydrate targets.

In recent years, nanopores have garnered attention as single-molecule sensors, especially in the fields of DNA and protein analysis^[9,57,58]. Nanopores are small holes in thin membranes that operate on a simple premise: when a molecule passes through a pore that is conducting ionic current, it causes a transient blockage in that current that is characteristic of that molecule. Previous work has demonstrated that solid-state nanopores can sense molecules such as heparin^[59] and hyaluronic acid^[60], and xylan^[14]. However, solid-state nanopore-based sensing still faces challenges^[61]. Events are often too fast for the measurement electronics to properly resolve them^[17], and target molecules may be too weakly charged for detection via voltage-driven passage through a nanopore^[17,18]. There remains a need to improve nanopore-based analytical techniques to address these challenges, but first the dynamics of passage and the forces acting on carbohydrates must be better understood.

The role of electrophoresis (EP) and electroosmosis (EO), which refer to the electric field driven process by which charged molecules are driven through a medium, and the flow of liquid dragged by counterions to a charged surface set in motion, respectively, has been well

established for DNA translocations through solid-state nanopores [62]. For proteins, the interplay between electroosmosis and electrophoresis can significantly affect the kinetics of capture and passage[63–69] because of the non-uniformity and weaker charge of their structures.

In this work, we aim to improve nanopore-based sensing methods and to better understand the dynamics of voltage-driven passage of carbohydrates. To do this, we explore the relationship between translocation characteristics and molecular structure to find optimal operating conditions. We also investigate the nature of a prototypical carbohydrate translocating through a solid-state nanopore while varying the relative influence of electrophoretic and electroosmotic forces. Heparin was selected because of its practical use as an anticoagulant[70], the recognized challenges associated with its complete analysis[71], and the need to identify contamination with oversulphated dermatan sulphate, which can lead to serious adverse reactions[72].

Since the direction and strength of electrophoretic and electroosmotic forces are dependent on molecular charge, applied voltage, and nanopore wall surface charge density[68], we conducted a series of experiments in conditions of varying pH to provide control over both molecular and pore wall surface charges. We provide details of the pores and conditions used in the methods section. We estimated the conformations and shapes of heparin molecules as they pass through the pore by comparing our experimental results with an analytical conductance model, described in detail elsewhere[73]. We also studied the kinetics of voltage-driven passage of heparin as a function of pH. Throughout, we consistently find that overall dwell times are extremely fast, often on the scale of μs .

2.2. Materials and Methods

2.2.1. Nanopore fabrication

We used 5 mm x 5 mm chips with 200 μm -thick silicon frames purchased from Norcada for this study. The membrane has a 40 μm x 40 μm area containing a free-standing silicon nitride (SiN) membrane with an oxide underlayer and a thickness of 12 nm $\pm$ 2 nm. While thicker membranes could result in slower translocation, 12 nm membranes were chosen in favour of retaining signal amplitude in cases of smaller molecules like heparin, as the absolute strength of the signal increases with decreasing membrane thickness and as the pore diameter approaches the molecular size[73–75] Flow cells were designed and 3D printed in-house. These flow cells provide fluidic access to both sides of the membrane alike those presented in previous work[19]. See SI Figure 2.6 for the power Spectrum density (PSD) of a device. We fabricated pores using the controlled breakdown (CBD) method, following best practices previously published[19,76]. After fabrication, pores are usually relatively small, ranging from 1 nm to 3 nm according to I-V measurements and assuming a cylindrical pore model[74,75], and must be enlarged to the desired size following previously established protocols using alternating pulses of intermediate voltage to controllably enlarge the pore[20,77]. Nanopores used for experiments were 5 nm $\pm$ 1 nm in diameter based on I-V measurements unless otherwise noted.

2.2.2. Electrolyte and Analyte Preparation

We fabricated and enlarged pores in 1 M KCl and 3.6 M LiCl solutions, respectively, and buffered at pH 8 with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Analyte mixtures were made by dissolving powdered heparin purchased from Sigma Aldrich (porcine intestinal mucosa H3149) in 3.6 M LiCl and 4 M KCl to a final concentration of 500 nM in both cases. For experiments in 3.6 M LiCl, at pH 8, the same solution as the

fabrication solution was used; at pH 5, 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) was used as a buffer, while at pH 4 and 2, solutions were unbuffered. pH measurements were taken for the pH 4 solution before and after the experiments to confirm stability with ± 0.1 . For experiments in 4 M KCl, the pH 8 solution was buffered with 10 mM HEPES, pH 6 with MES, pH 4 with 20 mM acetic acid and 4 mM sodium acetate, while pH 2 remained unbuffered. For oversulphated chondroitin sulphate (OSCS), powder was purchased from Sigma Aldrich (U.S. Pharmacopeia #1133580), and experiments were conducted in 4 M KCl solutions at a concentration of 500 nM, at pH 4, 6, and 8, using the same buffers as above.

2.2.3. Data Acquisition and Analysis

We acquired data using the Chimera VC 100, which has a sampling rate of 4.17 MHz and a full bandwidth of 1 MHz. A pair of Ag/AgCl electrodes is interfaced between the VC 100 unit and the solution to both apply voltage and measure the ionic current through the pores. In all experiments, the voltage applied was ± 200 mV. The choice of voltage of ± 200 mV is adopted from many previous similar solid-state nanopore studies[31,66,78–80]. Data was low-pass filtered with a digital 8-pole Bessel filter with a cutoff of 200 kHz to provide sufficient signal-to-noise ratio (SNR) for analysis. We analysed the data using Nanolyzer, developed by Northern Nanopore Instruments (now Oxford Nanopore Technologies), defining an event as a deviation from the baseline of 6.5 times its local root-mean-square (RMS) noise.

We measured the average molecular weights of heparin & OSCS using size exclusion chromatography on an Agilent 1260 Infinity II system with a Superose 12 10/300 GL column with phosphate buffered saline (PBS) as an eluant. A dextran ladder from Agilent enabled size comparisons between the heparin and OSCS samples (see SI Figures S4 and S5). Analyte detection was based on a refractive index detector.

2.3. Results and Discussion

2.3.1. Heparin Translocation Signals and Molecular Size

To investigate the translocation kinetics of heparin through a solid-state nanopore, we conducted a series of experiments with heparin solutions at different pH values to explore any differences resulting from different protonation states (Figure 2.1 a). Heparin is a highly acidic molecule with sulphate monoesters and sulfamido groups with a pK_a of approximately 1. It also contains weaker carboxylic acids (uronic acids), which can be partially protonated depending on the pH ($pK_a \approx 3$)[81]. As some have linked the protonation states of heparin to structural changes (e.g. loss of helical structures at a lower pH[82]), and the charge of the heparin molecules might interact with the pore surface, we hypothesized that modifying the pH might result in slower translocations and allow for more accurate measurement of the blockage signature. Previous estimates have shown that SiN has an isoelectric point around pH 4-5[83].

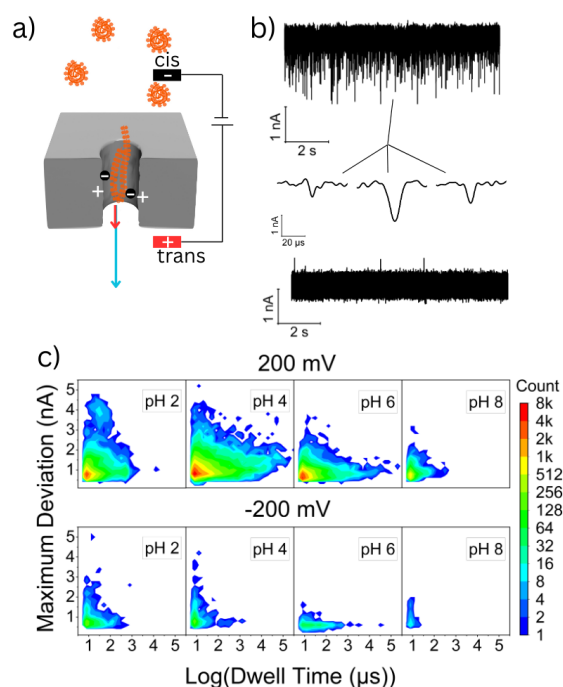


Figure 2.1 Nanopore heparin sensing setup, representative traces and events, and heat maps of scatter plots at different conditions. (a) A schematic of the experimental setup. Heparin solution was added to the electrically grounded side. **(b)** An example of a 1-second trace of heparin translocations 4 M KCl 10 mM HEPES pH 4, through a 5.4 nm pore at +200

mV (top) and -200 mV (bottom). Inset: examples of specific events. The signal is digitally low-pass filtered at 200 kHz. **(c)** Heat maps of scatter plots of heparin translocations showing maximum current deviation versus dwell time in log scale at different pH values. All heat maps show events over the course of 1,200 s of data acquisition, except for pH 8 with only 600 s of data, highlighting significant differences in the number of events, N , and thus in event rate. For +200 mV: pH 2 ($N \sim 14,000$), pH 4 ($N \sim 36,000$), pH 6 ($N \sim 24,000$), pH 8 ($N \sim 2,800$); for -200 mV: pH 2 ($N \sim 1,200$), pH 4 ($N \sim 600$), pH 6 ($N \sim 700$), pH 8 ($N \sim 240$).

The ionic current trace and individual events of heparin translocating through a 5.4 nm ± 0.4 nm pore in a 12 nm ± 2 nm thick membrane pore in 4 M KCl, pH 4 are shown in Figure 2.1 b. Figure 2.1 c shows the heat maps of scatter plots of maximum deviation against dwell time on a log scale. Maximum deviation refers to the current difference between the baseline and the single data point that is farthest away from the baseline within an event (i.e., maximum blockage level in the event + noise), and as such, is sensitive to the level of noise and the applied low-pass filter. This metric is used instead of more traditional fits when events are fast and not conducive to accurate fits, as distortions arise, and events are missed when the duration of translocation events is similar to the response time of the electronics of the system[84]. All sub-panels of Figure 2.1 c contain events from the same 5.4 nm pore for 4 different pH values and both opposite applied voltages. Here, we define an event as beginning whenever the signal deviates by > 6.5 standard deviations from the baseline.

In Figure 2.1 c, we see that under positive applied voltage (+200 mV), population variations at different pH levels are evident (pH 2: $N \sim 14,000$; pH 4: $N \sim 36,000$; pH 6: $N \sim 24,000$; pH 8: $N \sim 2,800$; where N is the number of single-molecule events recorded in 1,200 seconds, except for pH 8 which was recorded for 600 seconds). Most heparin events have a maximum deviation between 300 pA and 1000 pA, with a median maximum deviation in the range of 600 to 750 pA for all pH values except for pH 4, which has a maximum deviation of 870 pA. SI Figure 2.7 shows the histograms of maximum deviation distribution at this positive voltage for all four pHs. Under negative applied voltage (-200 mV), electrophoresis opposes

translocation of negatively charged molecules, and consistent with this, we observe few translocations at all pH values (pH 2: $N \sim 1200$; pH 4: $N \sim 600$; pH 6: $N \sim 700$; pH 8: $N \sim 240$). The events we observed at negative bias are most likely results of simple diffusion. The flux of analytes due to free diffusion is given by the Smoluchowski equation[85]: $J = 2\pi c D r_p$. For our conditions, this evaluates to 1-10 molecules/s, and our experimental event rate at the negative applied voltage does not exceed this theoretical estimation (see also Figure 2.8 for current traces). Here, concentration $c = 500$ nM, the radius of the pore, r_p , is 2.5 nm, and the diffusion constant of heparin is taken as $1.2 \times 10^{-12} \text{ m}^2/\text{s}$ [86].

To relate the observed blockage amplitudes to molecular shape to better understand the conformation in which these heparin molecules pass through the nanopore, we used an analytical model of the pore conductance that accurately accounts for the contribution of the pore access resistance, and available as a webapp (<https://www.tcossalab.net/signalGenerator>), that can calculate expected nanopore signals from different molecular geometries[73]. In a nutshell, this signal generator uses an analytical framework to predict the measured signal caused by a particular analyte shape passing through a pore of a given geometry. The model can accurately predict the molecular signatures as a function of the distance of the pore by correctly modelling the region outside the pore, which is reminiscent of the blockage signal versus time recorded experimentally (assuming a constant velocity). The details of the model used are the subject of a previous publication. Here, we model heparin molecules with different conformations as cylinders of different radius and length. The pore is modelled as a cylindrical opening with a radius of 3 nm and a thickness of 12 nm. A dwell time of 10 μs was used for the simulation. In Figure 2.2 a, the compact molecule (stretched blob) is estimated as having a radius of 2.9 nm and a length of 5 nm, and in 2b, the snake-like shape (series of smaller blobs) is estimated as having a radius of 1.5 nm and a length of 10 nm. In 2c, the fully elongated linear

chain has a radius of 1 nm and a length of 19 nm, and the linear heparin chain in 2d has a radius of 0.5 nm and a length of 38 nm.

The heparin sample used here had an average molecular mass of 28 kDa with a polydispersity index of 1.64, as determined by HPLC (see Methods section 2.3, Figure 2.9, 2.10). Heparins are polydisperse polymers, and a sample molecular weight can range between 5000-50000 Da[87]. It is composed of iduronic acid and glucosamine in their pyranose form [88]. The disaccharide unit has a molecular mass of 0.37 kDa and thus consists of approximately 75 units considering an average heparin mass of 28 kDa. A previous study has found that one pyranose ring in its relaxed state has a diameter of 0.45 to 0.54 nm, depending on the chain conformations between 4C_1 and 1C_4 [89]. Therefore, we can approximate heparin as a flexible cylindrical chain with an equivalent diameter of ~ 1 nm and an end-to-end distance of ~ 38 nm. Assuming our polymer executes a 3D random walk with a persistence length of 2.1 nm[90], the radius of gyration of the heparin coil is expected to be 3.9 nm[91], but previous measurements have also found them to be around 3.2 to 3.5 nm[90]. We therefore do not expect heparin to translocate as a densely packed blob through a pore with a radius of ~ 2.5 nm (diameter ~ 5 nm) used here.

Similarly, starting from the opposite extreme of possible conformations, heparin can be modelled as a stretched linear chain. If we simulate translocation of a fully extended molecule, the predicted signal is much shallower than our experimental observations, suggesting that molecules are not fully extended when translocating through ~ 5 nm pores either, since our deeper observed blockages imply the presence of more than a single molecular chain in the pore at any given time. Figure 2.2 shows this situation schematically.

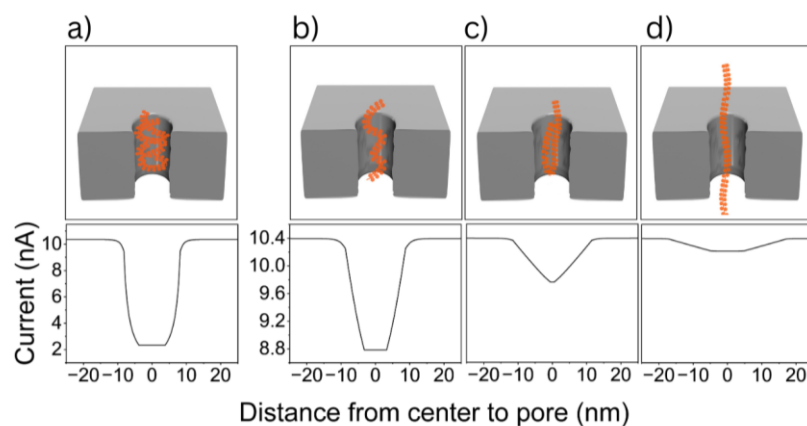


Figure 2.2 Possible heparin conformations passing through a nanopore and simulated signals. (a) Illustration of a heparin molecule passing through a solid-state nanopore as a stretched blob, producing a deep signal that deviates from the baseline by ~ 8 nA, as calculated by our conductance model. (b) Illustration of a heparin molecule passing through a solid-state nanopore as a series of smaller blobs, producing a signal that fits the observed signatures of some of the deeper heparin events. This is modelled as a cylinder with a diameter of 3 disaccharide units in our Signal Generator tool. (c) Illustration of a heparin molecule passing through a solid-state nanopore as a folded chain, producing a shallow signal that fits the profile of most heparin translocation events. (d) Illustration of a heparin molecule passing through a solid-state nanopore as a fully elongated linear chain, producing a signal that would be too shallow to explain the experimental results. Note that the y-axis scale for the plot in (a) differs from that in (b), (c) and (d).

Although short, charged polymers may adopt a rod-like conformation due to electrostatic repulsions, counterion condensation could explain the folded chain conformation we observed, as cations act like shields to sulphate groups in highly concentrated salts. Extreme bending of molecules during solid-state nanopore translocation is well established. As previous studies show, ssDNA and dsDNA with much longer persistence length than heparin translocate through nanopores in folded configurations [78,80,92]. We conclude that heparin molecules are more likely to pass through the nanopore as a folded chain or as a series of smaller blobs, as depicted in Figures 2b and 2c, but the high degree of variation observed in the signals is consistent with a high degree of flexibility[80], and the spread in molecular weight. Previous work suggests that, for charged molecules, some elongation is expected on approach to the pore[78,93], though we note that numerous molecular configurations can give rise to the same

maximum deviation. A matching value between the simulated blockage for a particular conformation and experimentally observed blockages does not necessarily mean that the signal was generated by a molecule translocating in that conformation. Nonetheless, assuming a partially linearized translocation enforced by the confinement of the nanopore, we found that a cylindrical molecule with a diameter of 2 nm generates a signal that reasonably approximates a typical experimentally observed heparin translocation event, with a predicted maximum deviation of 500 pA.

Heparin molecules can also take the form of a smaller blob, as Figure 2.2 b shows schematically. We modelled this conformation as a long cylinder with a diameter of three disaccharide units, generating a signal that is approximately 1600 pA. Such signals fit the profile of the deeper events we observed. This suggests, unsurprisingly, that heparin does not translocate through the nanopore as a fully coiled blob as it is found in the bulk, but rather as a partially folded chain or one with a series of smaller blobs corresponding to 3 disaccharide units, as schematically shown in Figures 2b and 2c.

2.3.2. Heparin Translocation Kinetics and Event Rates

As noted previously, heparin translocations show different event detection rates at different pH values under a positive 200 mV applied voltage. The motion of charged molecules inside a nanopore is governed by a balance of electrophoresis and electroosmosis. The direction and strength of electrophoretic and electroosmotic forces depend on the applied voltage, the charge of the molecules, and the surface charge of the nanopore. Varying the pH was intended to change the charge state of the nanopore walls to affect the magnitude of the EOF and the net charge of heparin to affect the magnitude of the EP force, while changing the polarity of the applied voltage was aimed at exploring which of the EO and EP forces dominates the kinetics of molecular transport. Based on the results presented in Figure 2.1 (and Figure 2.8), the

translocation of heparin appears to be dominated by electrophoresis. This is supported by theoretical estimates of the EO and EP velocities for a heparin blob passing through a charged channel (see SI section S6). Similarly to DNA, for which the phosphate backbone is almost always deprotonated at all pHs, heparin is strongly acidic, and the molecule acquires a negative charge as the sulphate group (SO_3^-) on the monomer are deprotonated[94]. Studies have found that heparin begins to be protonated below pH 3 and should have a reduced net negative charge at pH 2[95,96]. On the other hand, heparin should stay consistently negatively charged between pH 4 and 8[94–97]. This implies a steady level of electrophoretic force towards the *trans* side throughout the pH 4, 6, and 8 values, but a decrease at pH 2.

It is possible to quantify the charge of a nanopore at different pH values by measuring its zeta potential[68]. In a previous study, we measured the zeta potential of silicon nitride solid-state nanopores as a function of pH using the streaming current method[83]. It was found that, like other studies[98], the isoelectric point is 4.4 ± 1 . This implies that above pH 5, the surface has a negative zeta potential and a negative surface charge, resulting in an EO force opposing the EP force acting on the negatively charged heparin. This opposing force increases in strength with increasing pH as the pore walls become more negatively charged, while at pH 4, the electroosmosis is very weak, and below pH 4 would reinforce electrophoresis. We illustrate these scenarios in Figure 2.3.

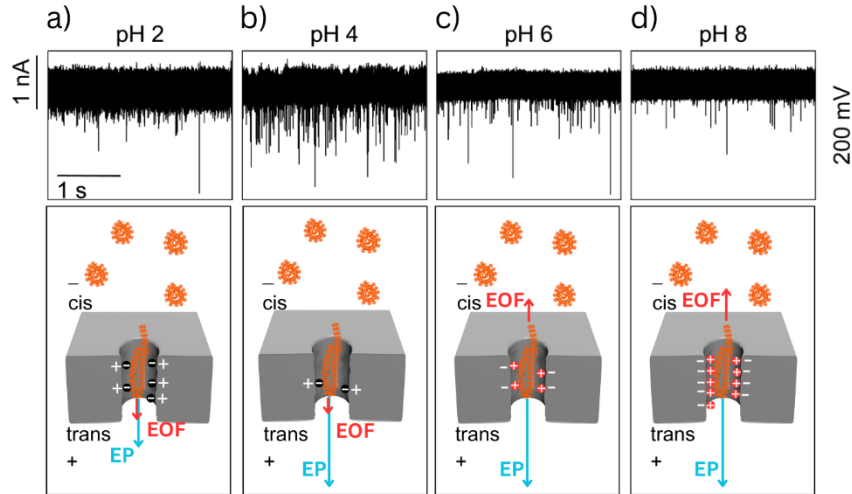


Figure 2.3 Force diagrams of a heparin molecule translocating through a nanopore at different conditions. (a) 3-second traces of heparin translocations at pH 2, electroosmosis (EOF) and electrophoresis (EP) point in the same direction, although electrophoresis is weaker than at higher pHs because heparin molecules lose negative charge in extremely acidic environments. (b) 3-second traces of heparin translocations at pH 4, electroosmosis and electrophoresis point in the same direction, while heparin is fully negatively charged, the net driving force is at maximum. (c) 3-second traces of heparin translocations at pH 6, electroosmosis is now opposing electrophoresis due to the change in the sign of the nanopore surface charge, causing a decrease in net force. (d) 3-second traces of heparin translocations at pH 8, electroosmosis increases even more with a more negatively charged pore wall, further weakening the net force. All data at 4M KCl in the same 5.4 nm pore as in Figure 2.1.

At pH 8 (Figure 2.3 d), the EO force opposes EP transport, and we observe few events. As we decrease the pH to pH 6, zeta potential is reduced, lowering the magnitude of the EOF opposing the electrophoretic driven passage of heparin, so that the event rate increases (Figure 2.3 c). At pH 4, the surface charge of the nanopore is almost neutral (very weakly positive) and the EO force now almost vanishes or points toward the EP force, while the charge of heparin remains negatively charged and unchanged, resulting in maximal event rate (Figure 2.3 b). At pH 2, although the EO force is stronger, heparin loses some of its negative charge[94–97], resulting in a weaker EP pull and thus a lower net force acting on the molecule, illustrated by the reduced observed event rates (Figure 2.3 a). We calculated the event detection rates at each pH using the inter-event time distribution[99] during 1,200 s recordings of the ionic current (see Figure 2.4 a).

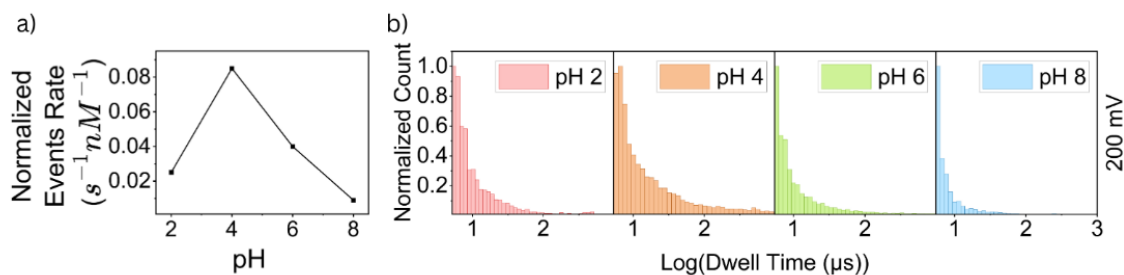


Figure 2.4 Event rate and dwell time histograms of heparin translocations at different conditions. (a) The concentration normalized event detection rate of heparin translocations at +200 mV applied voltage, showing non-monotonic behaviour as a function of pH. **(b)** Normalized dwell time histograms of heparin translocations on a log scale, showing the tail of otherwise under resolved distributions. Both (a) and (b) showcase the same dataset found in Figure 2.3 a, with the same low-pass filter at 200 kHz for analysis. The time distribution of negative polarity is included in SI Figure 2.12.

Figure 2.4 b displays the normalized histograms of log-scaled event dwell times at different pH. Most events translocate faster than the ability of our hardware to resolve them, as almost all events are faster than 100 μs , and the median dwell time observed is $<10 \mu s$ in all conditions. Although our events were longer than twice the rise time, the internal event structure that could report on molecular differences at these pHs cannot be resolved. At this timescale, the signal starts to be attenuated by our low-pass filter. Even with the full 1 MHz bandwidth of our measurement amplifier, these histograms only capture the tails of the full distribution of the heparin translocating in a 5.4 nm pore in 4 M KCl. However, there is limited ability to increase the 200 kHz cutoff frequency, since heparin events are inherently shallow, and this filter is necessary to reduce the noise sufficiently for analysis. To capture more of the distribution and better understand translocation kinetics, either a significant reduction of noise must be achieved (for example, through chip capacitance reduction[100]) or methods to slow down the heparin passage (like binding the target molecules to another larger molecule to achieve a deeper blockage and add more drag[18]).

At present, we can draw no quantitative conclusions about the dwell times, given that we are only able to sample the tail of the distribution. The analysis based on the blockage depth is valid for the events observed but may not be effective across the entire population, since some events are missed. While we found that heparin translocated too fast to fully resolve, we note that several groups have suggested methods by which to slow down molecular translocations. These include various salt conditions[101–103], narrower pore geometry[92,104,105], interfacing the pore with gel media[106,107] and modification of the molecule or pore surface[106,108,109], but the slowdown factors observed, ranging from 2–10× are unlikely to be sufficient to fully resolve the entire distributions in this case. Use of reduced voltage, to reduce speed during passage, results in capture rates too low to be practical in most experimental conditions. This suggests that researchers conducting experiments with heparin should be cautious about drawing conclusions relating to the details of translocation events, as even state-of-the-art electronics are inadequate to fully resolve these distributions without additional work to slow down molecular passage.

We performed a similar pH sweep in 3.6 M LiCl solutions to study the effect of the counterions on the kinetics of passage, and the overall trend is comparable to 4 M KCl on two additional ~5 nm pores. Key characteristics like dwell time and blockage depth have no distinct differences between the two salts. We include all related analyses, including maximum deviation versus log scaled dwell time scatterplots and log scaled dwell time histograms in the Supplementary Information Figures S8 and S9. A voltage sweep in 1 M KCl pH 8, with a 7.5 nm pore, is also included in Supplementary Information Figure S10.

Another factor that affects the electro-osmotic flow response is the concentration of the salt solution that changes the zeta potential of the nanopore surface. In our previous study[83], we compared the zeta potentials of the nanopore surface in KCl solutions at pH 8 in different concentrations and confirmed that the zeta potentials increase as concentrations decrease.

Because heparin translocation is primarily governed by electrophoresis, performing experiments at lower salt concentrations presents distinct challenges. Although the anticipated increase in EOF opposes EP motion at $\text{pH} > 4$, it appears insufficient to achieve the >10 -fold deceleration required to fully resolve the distribution of passage times. Conversely, at $\text{pH} \leq 4$, an increase in EOF would be counterproductive, as it would act synergistically with electrophoresis to accelerate translocation times. A lower salt concentration also means lower solution conductivity, which is directly proportional to our signal amplitude. As heparin already produces fast and shallow signals, maintaining a sufficient SNR for detection at low concentrations may be challenging.

2.3.3. OSCS and Heparin Contamination

Oversulphated chondroitin sulphate (OSCS) is a type of polysaccharide that contaminated heparin to deadly effect in 2008[110]. OSCS has a higher degree of sulfation compared to its counterpart, which is the naturally occurring chondroitin sulphate [110]. Although heparin and OSCS share similar chemical structures, OSCS-contaminated heparin triggers severe allergic reactions and hypertension in the human body and could potentially lead to death[35]. We therefore sought to study the translocation characteristics of OSCS with our nanopore system, as has previously been done[59].

The OSCS sample tested here has an average molecular weight of 18kDa with a polydispersity index of 1.29 (see Figure 2.9, 2.10). Figure 2.5 a shows heat maps of maximum deviation against log scaled dwell time for OSCS translocations. Heparin and OSCS both exhibit similarly shallow blockage depths and translocate extremely fast, with comparable event rate responses to pH (see Figure 2.15). To assess whether these two molecules could be differentiated, we conducted an experiment to pass heparin, OSCS, and a heparin solution with a 30% mixture of OSCS through the same 5.1 nm pore. Figure 2.5 b shows a heatmap

comparison between these three scenarios at pH 4, with maximum deviation against log scaled dwell time.

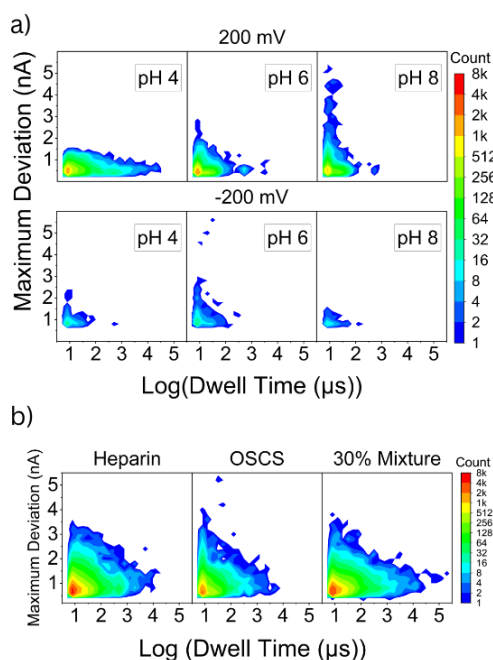


Figure 2.5 Heat maps of scatter plots of OSCS, and comparison between heparin, OSCS, and their mixture. (a) Heat maps of scatter plots of OSCS (oversulphated chondroitin sulphate) translocations in 4 M KCl showing maximum current deviation versus dwell time in log scale at different pH values. All experiments at positive voltage were done in the same $5.6 \text{ nm} \pm 0.4 \text{ nm}$ pore, whereas all experiments at negative voltage were done in another $5.2 \text{ nm} \pm 0.4 \text{ nm}$ pore. **(b)** Heat maps of scatter plots comparison between heparin, OSCS, and a 30% mixture (1:3 OSCS volume in heparin) at pH 4 at +200 mV in a $5.1 \text{ nm} \pm 0.4 \text{ nm}$ pore. All figures show events over the course of 1,800 s of recording.

We observed no distinctive features in the scatter plot to suggest that we could distinguish these two molecules despite the high bandwidth of our recording relative to previous attempts. Unlike a previous study[59], we did not see significant differences between OSCS and heparin passage signatures; however, as already discussed, a majority of events seem to be missed due to bandwidth limitations, and differences in behaviour may be present but unmeasurable at this bandwidth (see Figure 2.16). While improved time resolution could reveal more about molecular conformations, allowing them to be differentiated, higher bandwidth, reduced passage speed, and an improved signal-to-noise ratio would be needed than

is possible with our current hardware. We also note that our experiments used a different source of heparin with a larger molecular weight (28 000 Da vs 16 000 Da) as well as polydispersity (1.6 vs ≈ 1.3) (see Figure 2.9, 2.10).

2.4. Concluding Remarks

We have shown that most heparin molecular translocations through solid-state nanopores are fast relative to the ability of state-of-the-art electronics to resolve them. We have also demonstrated and established through force analysis that transport of these molecules is highly dependent on the interplay between molecular charge, nanopore surface charge, pH, and polarity of the applied voltage. A clear conclusion from this work is that to differentiate these molecules reliably with a solid-state nanopore will require a combination of slower translocation, higher bandwidth, and improved signal-to-noise ratio.

Other groups have developed several techniques that could be explored to address the passage speed challenge. One approach would be to attach a tag, a molecule that can bind to heparin[111], to increase the blockage depth. Lectins are excellent binding materials for polysaccharides[112] and could serve as a tag to slow heparin down during translocation. Another strategy could be to include a chemically reactive site inside the pore[113] that binds to translocating molecules transiently, creating a longer interaction with the pore. Boronic acid is one such coating which reversibly binds to hydroxyl moieties of carbohydrates[114,115].

2.5. Supporting Information

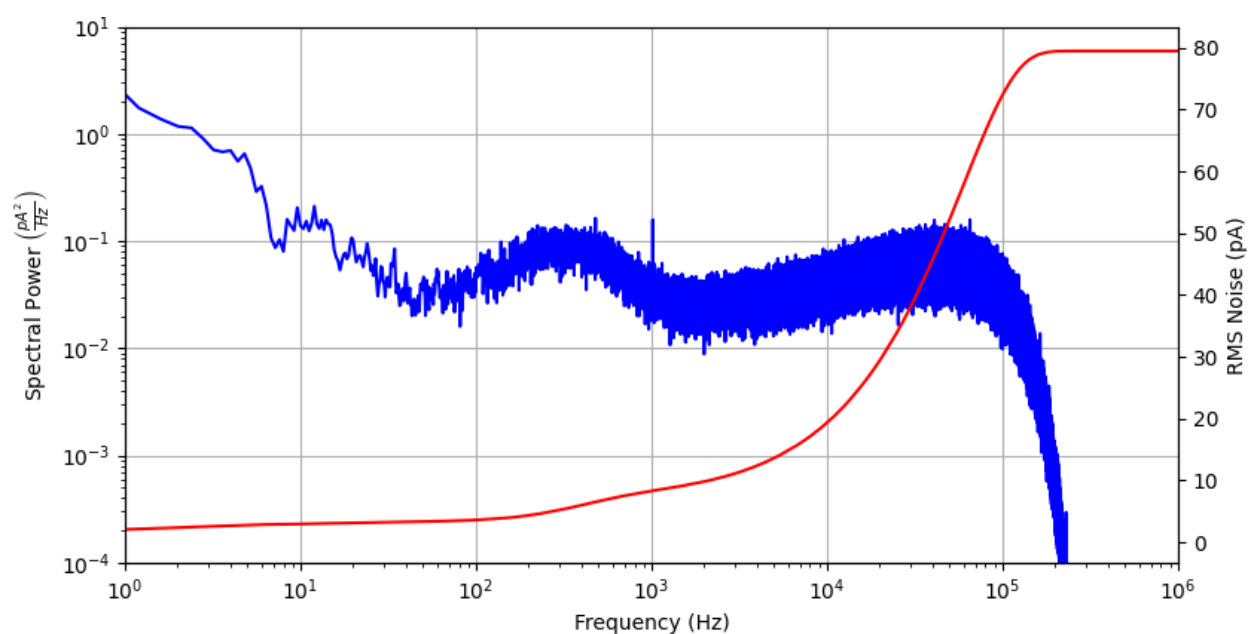


Figure 2.6 Power spectrum density (PSD) for the pore used in experiments described in Figure 2.1, which is $5.4 \text{ nm} \pm 0.4 \text{ nm}$ in a $12 \pm 2 \text{ nm}$ thick membrane. Measurements taken at 200 mV at a low-pass filter of 200 kHz.

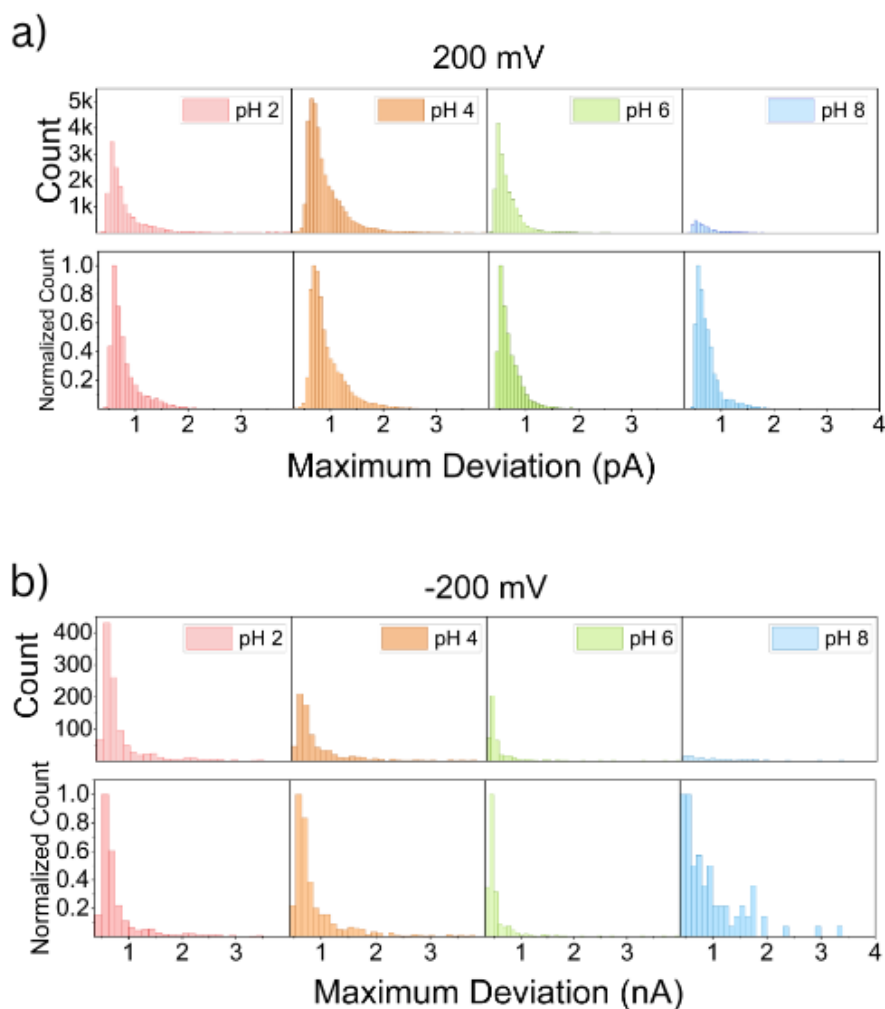


Figure 2.7 Histograms of maximum deviation data in Figure 2.1. a) The maximum deviation histograms and the normalized histograms of heparin translocations through a 5.4 nm pore at +200 mV (in 4 M KCl), showing that the variation of the detection rate and that the distribution of events mostly consists of shallow events at < 1000 pA. **b)** Histograms and normalized histograms demonstrating data taken at -200 mV at the same conditions and in the same nanopore. Data at a) and b) is the same data shown in Figure 2.1 c, with the same low-pass filter at 200 kHz for analysis.

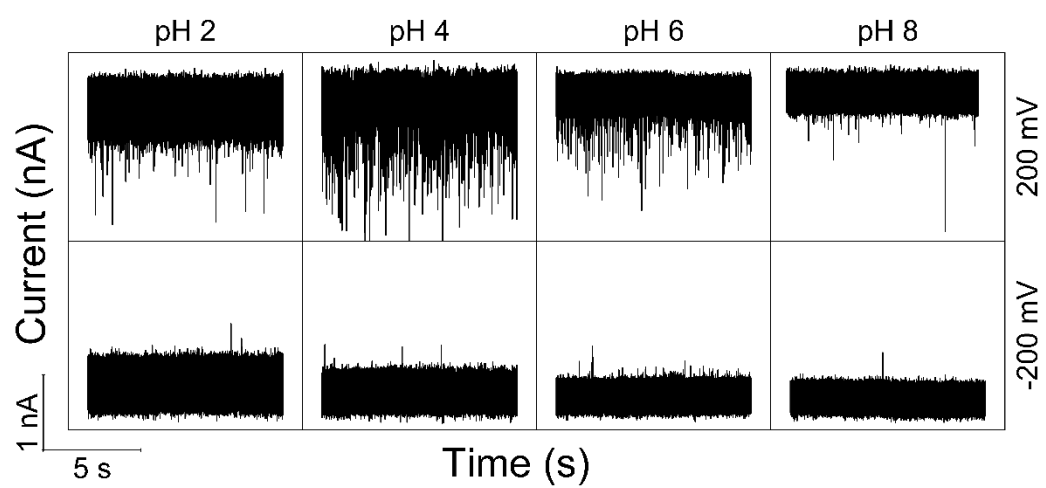


Figure 2.8 The 10 seconds traces of all experimental conditions showcased in Figure 2.1.

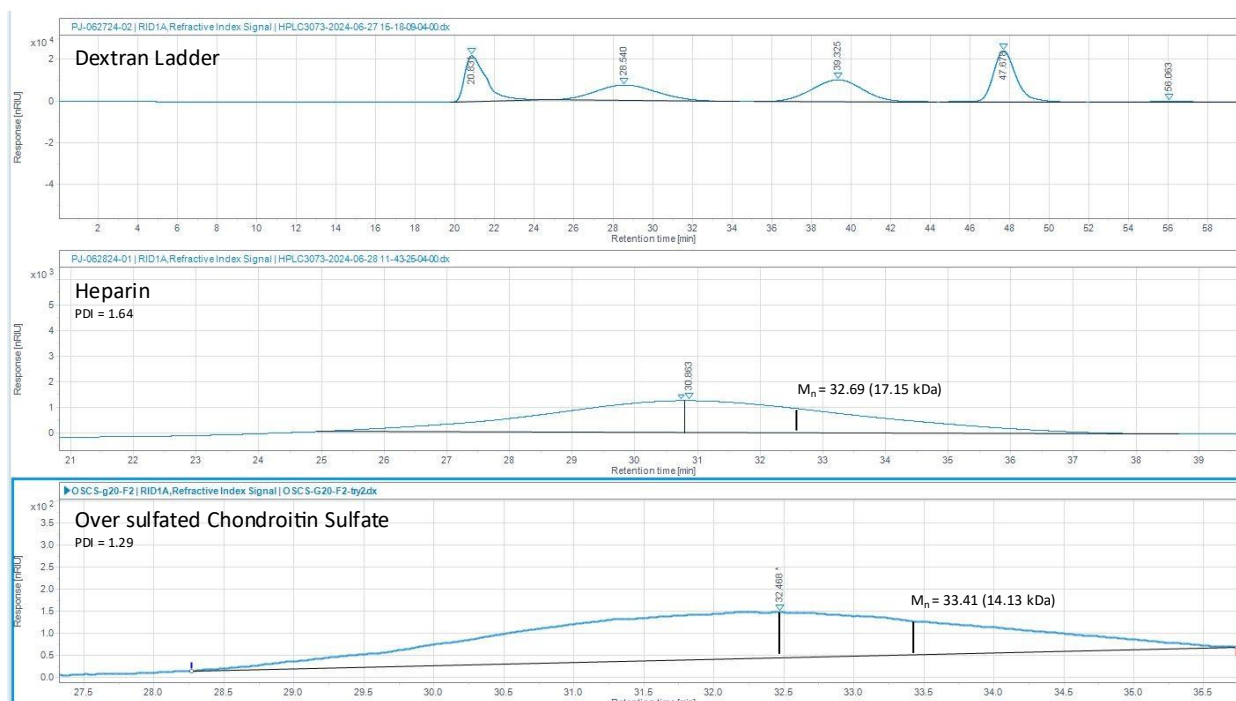


Figure 2.9 The size exclusion HPLC traces of the three samples (dextran ladder, heparin, and OSCS). Polydispersity indexes (PDI) have been calculated based on weight-average and number-average molecular weights attained from size exclusion traces.

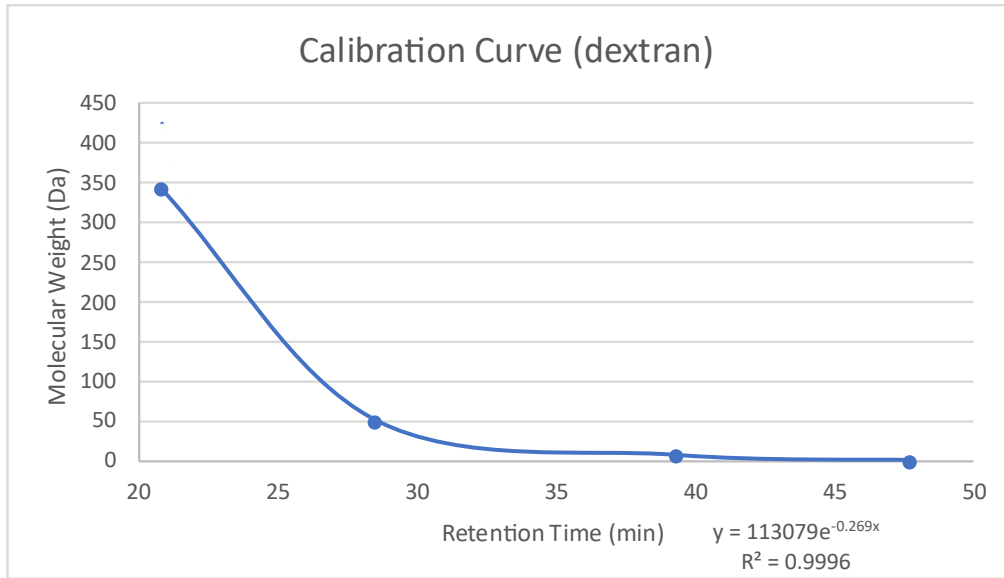


Figure 2.10 The calibration curve of the dextran ladder used to determine the sizes of OSCS and heparin.

Section S1: The electroosmosis velocity can be expressed as $v_{EO} = \frac{\epsilon_r \epsilon_0 V}{\eta L} |\zeta_{pore}|$, where ϵ_r is the relative permittivity (dielectric constant), which can be reduced for water in confined geometries, ϵ_0 the permittivity of free space, V the applied voltage, L the membrane thickness (or the electrostatic length of the pore), and ζ_{pore} the pore zeta potential (related to surface charge density). The electrophoresis velocity can be expressed as $v_{EP} = \mu_E \frac{V}{L}$, where μ_E is the electrophoretic mobility. For our system can be estimated to be $v_{EO} \lesssim \pm 0.01 \text{ m/s}$, depending on the pH, while $v_{EP} \sim \pm 1 \text{ m/s}$.

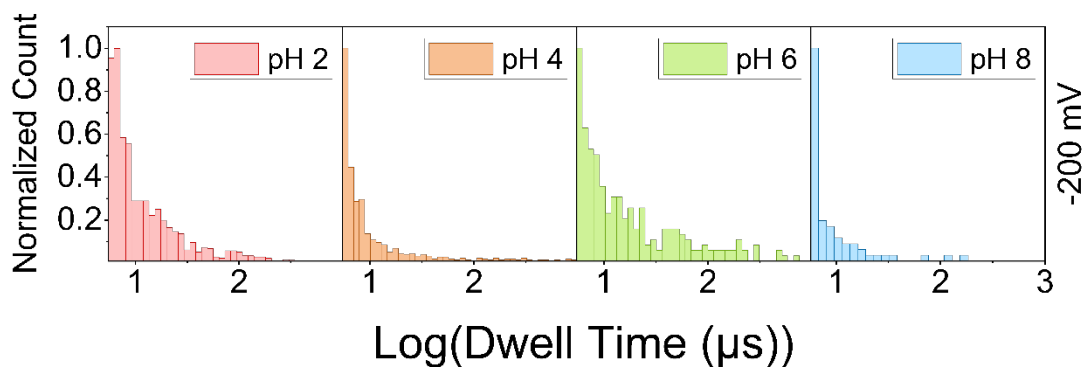


Figure 2.11 The dwell time histograms in log scale of heparin translocations through a 5.4 nm pore at -200 mV (in 4 M KCl). This is the same data demonstrated in Figure 2.1.

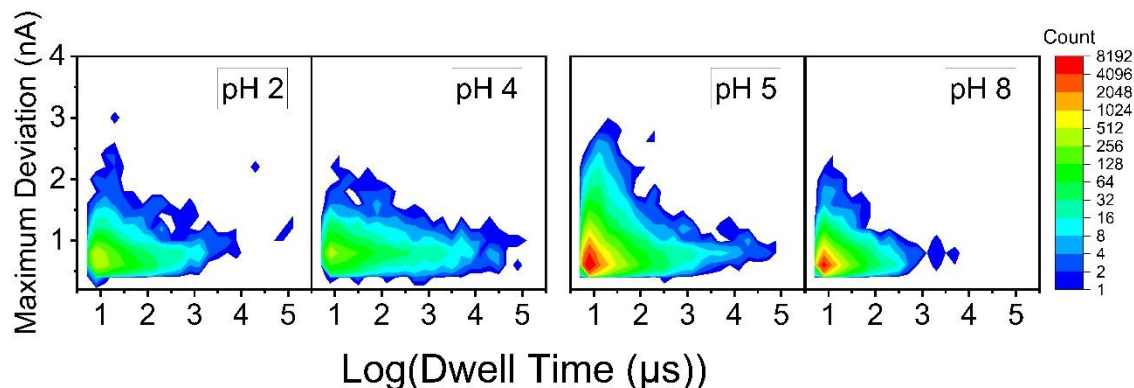


Figure 2.12 Heat maps of scatter plots of heparin translocations in 3.6 M LiCl at 200 mV at different pH values. All heat maps show events over the course of 1800 seconds of data acquisition. Note that because of clogging, only 900 seconds of data at pH 4 were acquired, analyzed and fitted. **Left** two panels (pH 2 and 4), experiments are performed on one pore, which is $5.0 \text{ nm} \pm 0.4 \text{ nm}$ in a $12 \pm 2 \text{ nm}$ thick membrane. **Right** two panels (pH 5 and 8), experiments are performed in another pore, which is $5.1 \text{ nm} \pm 0.4 \text{ nm}$ in a $12 \pm 2 \text{ nm}$ thick membrane. Signals were digitally low-pass filtered at 200 kHz.

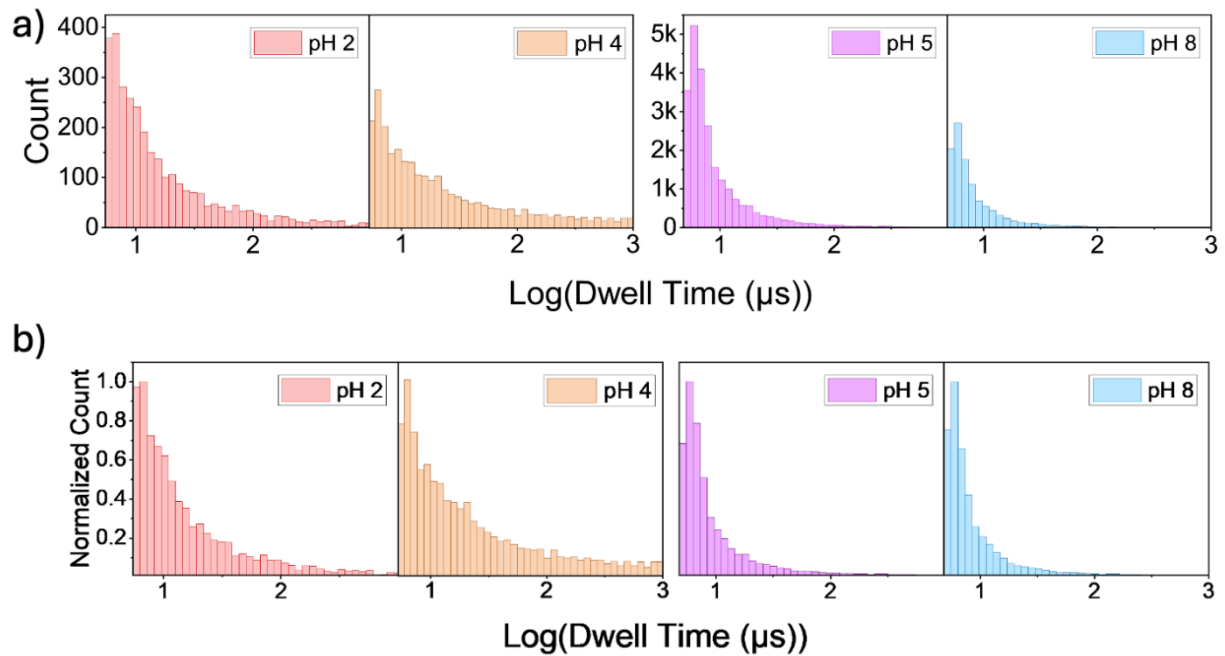


Figure 2.13 Dwell Time histograms of the experiments in Figure 2.12. a) dwell time histograms of heparin translocations in 3.6 M LiCl at 200 mV on a log scale, showing the tail of an otherwise under resolved full distribution. Left two panels (pH 2 and 4), experiments are performed on a $5.0 \text{ nm} \pm 0.4 \text{ nm}$ in a $12 \pm 2 \text{ nm}$ thick membrane. Right two panels (pH 5 and 8), experiments are performed on another $5.1 \text{ nm} \pm 0.4 \text{ nm}$ in a $12 \pm 2 \text{ nm}$ thick membrane. **b)** Normalized dwell time histograms of the same data as in a). This is the same dataset showcased in Figure 2.12, for 1800 s of data acquisition, with the same low-pass filter at 200 kHz for analysis.

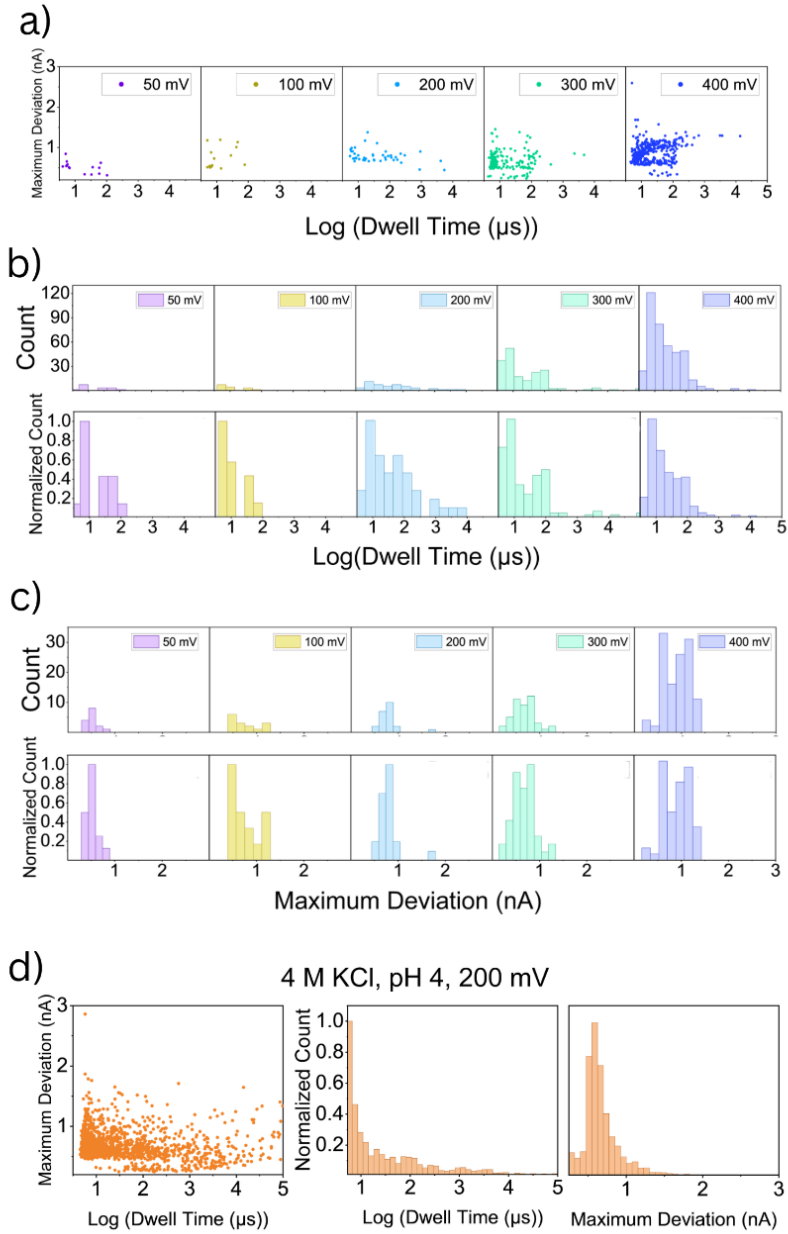


Figure 2.14 Scatter plots, histograms of dwell time and maximum deviation of heparin translocations in 1 M KCl, pH 8. **a)** Scatter plots of heparin translocations in 1 M KCl at pH 8 showing maximum deviation in nA versus dwell time in log scale at different voltages. All scatter plots show events over the course of 300 s of data acquisition, and experiments were performed in a $7.5 \text{ nm} \pm 0.5 \text{ nm}$ in a $12 \pm 2 \text{ nm}$ thick membrane. Signals were digitally low-pass filtered at 200 kHz. **b)** Histograms and normalized histograms of the dwell time data in a). **c)** Histograms and normalized histograms of the maximum deviation data in a), but only selecting events that have a dwell time longer than $50 \mu\text{s}$, to be fully resolved. **d)** Control group of heparin translocations in 4 M KCl at pH 4, 200 mV, events recorded for 300 s of data acquisition, showing a scatter plot, and histograms of both dwell time and maximum deviation. Note that in a) at 200 mV, the number of events $N \sim 50$, and in d) $N \sim 1,700$. These results yield limited additional insights to elucidate the transport mechanisms, as many events are still missed, meaning we are only observing the tail of the passage times distribution, preventing quantitative conclusions on passage speed. Nonetheless, we observe a reduced capture rate in 1 M KCl compared to the 4 M KCl condition—expected with a stronger opposing EOF. While passage appears slightly slower. At both 50 and 100 mV, $N \sim 15$, showing minimal capture.

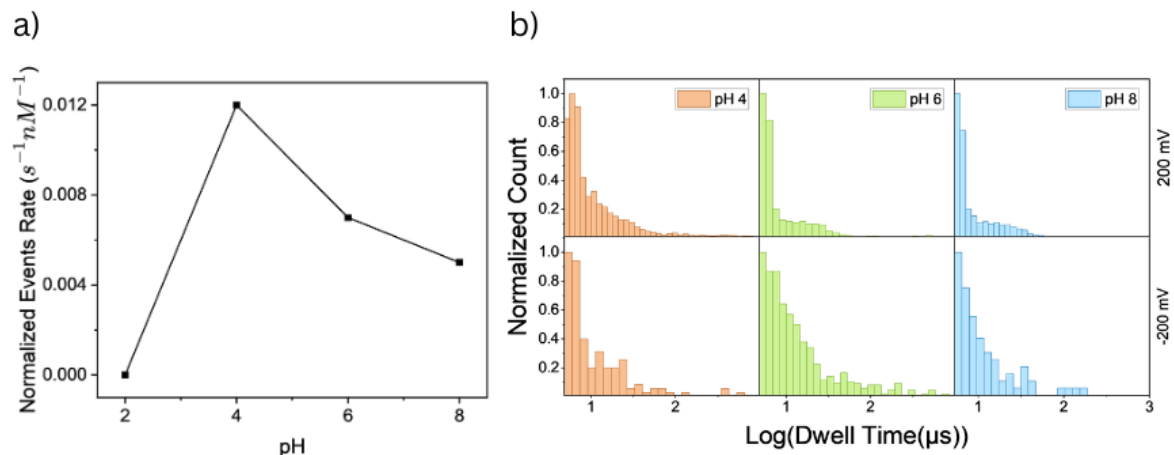


Figure 2.15 Event rate and dwell time histograms of heparin translocation in Figure 2.12. **a)** Concentration normalized event rate of OSCS translocations at 200 mV in 4 M KCl, showing non-monotonic behaviour as a function of pH. **b)** The normalized histograms of dwell time of OSCS translocations at positive and negative voltage, showing fast translocation time and under resolved population. All experiments at positive voltage were done in the same $5.6 \text{ nm} \pm 0.4 \text{ nm}$ pore, whereas all experiments at negative voltage were done in another $5.2 \text{ nm} \pm 0.4 \text{ nm}$ pore.

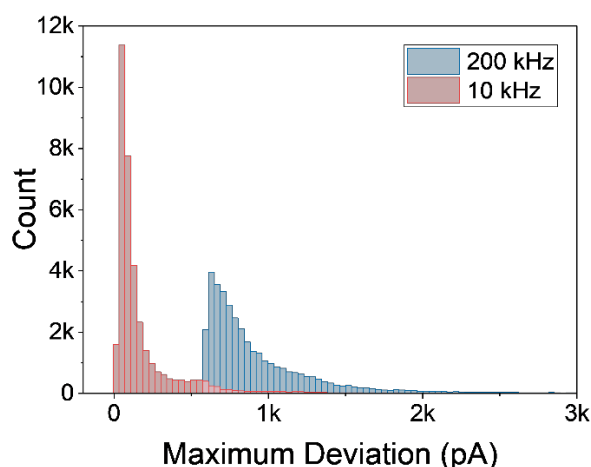


Figure 2.16 Comparison of histograms of maximum deviation when analysing at different cut-off frequencies. Histograms of the maximum deviation data of heparin translocations in 4 M KCl at pH 4, when analyzing at different Bessel filter cutoff frequencies, 200 kHz and 10 kHz, showing drastic differences in event depth.

In a previous study attempting to differentiate heparin and OSCS, it was found that these two molecules exhibit different behaviours when passing through a solid-state nanopore in 4 M KCl at pH 7 [Karawdeniya *et al. Surveying silicon nitride nanopores for glycomics and heparin quality assurance. Nat Commun* 9, 3278 (2018)]. In particular, the OSCS population contains a subset of events that have a much shorter dwell time and a deeper blockage, which we did not observe. Differences in sample dispersity could explain this discrepancy with our results. Another possibility is that the authors analyzed their data using a 10 kHz low-pass filter. Given that our dwell time distributions are unresolved even at 200 kHz, it is likely that many fast events were not resolved in that previous study. Low-bandwidth filters may be efficient when the pore quality is less than satisfactory, and the noise poses difficulties during data analysis. However, using an aggressive low-pass filter can attenuate the events as well as the noise. To illustrate this, here, we have taken our dataset of heparin translocations at pH 4, analyzed at a 200 kHz filter, and compared to the same events after applying a 10 kHz filter. The events are severely attenuated, demonstrating the distorting impact of low-bandwidth analysis.

3. Chapter 3. Conclusion

3.1. Pressure Flow Cell

As discussed in Chapter 2, heparin translocations are mainly electrophoretically driven. In principle, a platform that enables the application of a pressure gradient across the pore could create a hydrodynamic flow that opposes the electrophoretic force, thus slowing down the translocation. This is the subject of a research thesis by a current colleague in the lab, Mohamed Yassine Bouhamidi. He has utilized this method to slow down DNA and protein translocations with success. The setup he developed was based on the original flow cell design but contained key modifications to allow a pressure regulator to be connected to the cell with tubes, in order to apply a maximum pressure of 0.3 MPa. This setup is shown schematically in Figure 3.1[83,116].

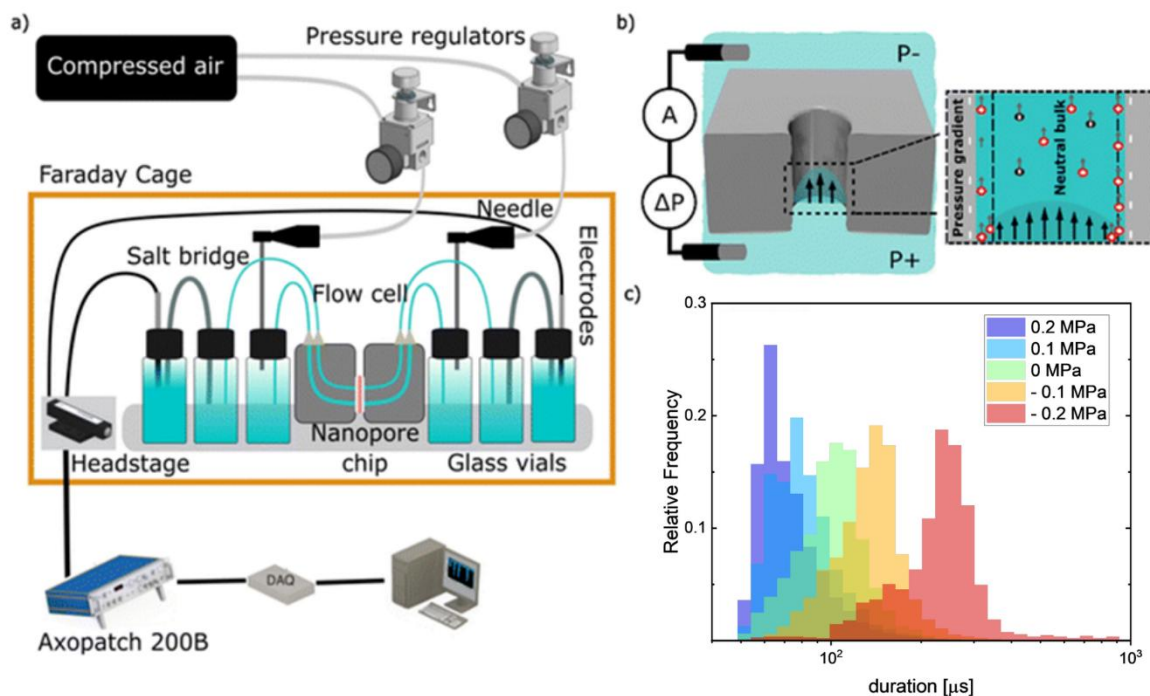


Figure 3.1 Pressure flow cell setup, workflow, and DNA pressure results. a) Setup of the pressure-controlled system, components utilized to achieve pressure application and current measurements, b) schematics of the nanopore system and the streaming current inside the pore. From *Unraveling clogging of solid-state nanopores by selective polymer coating: surface potential measurements and sensing of long single stranded DNA*, Bouhamidi et al., 2025, licensed under CC BY-NC 3.0. c) 2 kbp DNA translocations, histograms of dwell time, under

different pressures applied. From *Pressure-Voltage Modulation of Passage Dynamics for Enhanced Nanopore Biosensing*, Bouhamidi et al., 2026, submitted but not yet published.

Results have shown that this setup effectively applies a counterpressure to the nanopore system, as shown in Figure 3.1c, so as to change the timescale of 2 kbp DNA translocations noticeably[83]. Together with Yassine, we attempted to use this counterpressure to slow down heparin translocations.

In this experiment, in-house-made chips with a SiN thickness of 14 nm were used. The pore used to report the results shown in Figure 3.2 had an estimated diameter of ~ 5 nm. A solution of 500 nM heparin in 3.6 M LiCl, at pH 8, was injected, and a voltage of 200 mV was applied simultaneously with a 0.3 MPa counterpressure. Data acquisition was done using Axopatch 200B, and a 100 kHz low-pass filter was applied for data analysis.

We expected the heparin population to have a longer dwell time, similar to the DNA response in Figure 3.1c. However, as Figure 3.2 shows, heparin in 3.6 M LiCl, at pH 8, with or without the 0.3 MPa counterpressure, has almost identical populations. Disappointingly, there was no change in both timescale and blockage depth. While there were fewer events captured when pressure was applied, which was expected due to the opposing hydrodynamic force that reduced the capture, heparin signals remained fast and shallow, seemingly unaffected by the counterpressure. This contrasts with DNA or folded proteins that can be significantly slowed down in this way. It remains unclear why short carbohydrates do not feel the pressure-induced fluid flow, but operating conditions may need to be optimized to experience an effect. In future work, performing separate voltage and pressure sweeps could help identify if a counter pressure can be used for slowing down the kinetics of heparin passage through solid-state nanopores.

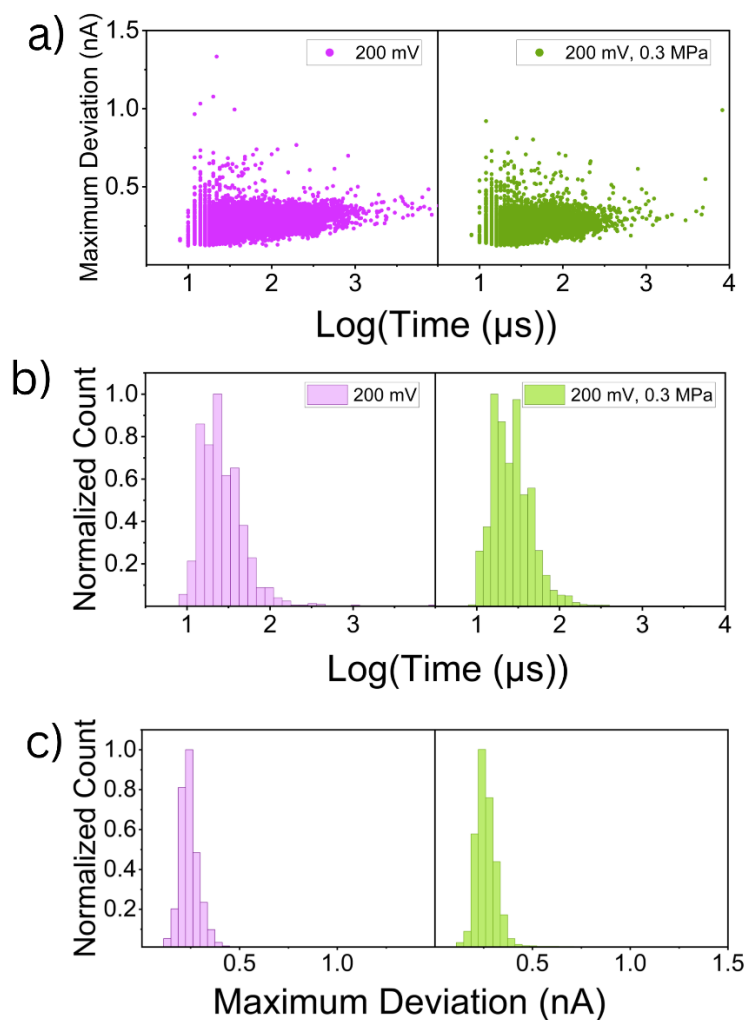


Figure 3.2 Scatter plots, histograms of dwell time and maximum deviation of heparin translocations, with and without counter pressure. **a)** Scatter plots of heparin translocations in 3.6 M LiCl at pH 8 showing maximum deviation in nA versus dwell time, with and without counter pressure, at 200 mV. Events shown 3600s of data acquisition. Experiments were performed in a $5.1 \text{ nm} \pm 0.2 \text{ nm}$ pore in a $14 \pm 2 \text{ nm}$ thick membrane. $N \sim 20000$ without counter pressure, $N \sim 10000$ with 0.3 MPa counter pressure; **b)** normalized histograms of the dwell time distribution; **c)** normalized histograms of the blockage depth distribution.

3.2. Lectin Binding

As previously reviewed, it has been shown that heparin could bind to protein molecules, and their translocation speed can be greatly reduced to better resolve the single-molecule events[48]. Other than FGF-1, a protein used by Thyashan et al.[48], it has also been revealed that lectins could bind to heparin molecules with a 1:1 ratio. Lectins are naturally occurring proteins, found in a wide range of living organisms, and are famously sugar-binding. Sandoval et al. published an article in 2019[117], showing that lectin, more specifically CLEC14A, can bind with heparin with a K_d of 0.45 $\mu\text{g/ml}$ in 1 M NaCl at pH 7.1. From CLEC14A product information, its molecular weight is determined to be around 70 to 80 kDa, based on Bis-Tris PAGE results. Using an estimation of 75 kDa, this binding affinity translates to 6 nM. They incubated lectin and heparin with a 9:1 concentration and measured that these two molecules bind within minutes[117].

As Figure 3.3 illustrates, the CLEC14A molecule is very small, with a width and length of 2 to 3 nm. It possesses positively charged regions consisting of certain amino acid groups that can bind to the sulphate groups in heparin molecules. Another study has found that lectin molecules have an isoelectric point between pH 4.5 and 5.5[118], and it is overall negatively charged in a more basic environment and positively charged in an acidic environment.

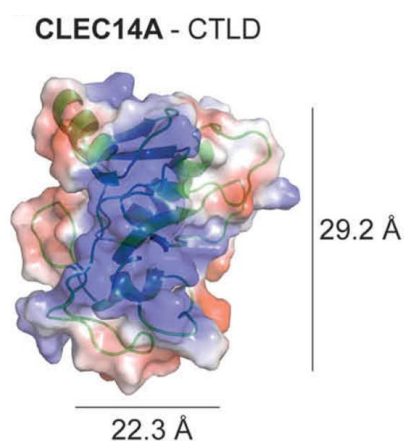


Figure 3.3 Illustration of a CLEC14A molecule, showing its size in angstroms. Colours represent polarity; red is the positively charged area, and blue is the negatively charged area.

From *Proteomics-based screening of the endothelial heparan sulfate interactome reveals that C-type lectin 14a (CLEC14A) is a heparin-binding protein*, Sandoval et al., 2019, licensed under CC BY 4.0.

Consequently, we postulated that lectin and heparin could bind and form a larger complex that would translocate through the nanopore more slowly due to the added drag and produce deeper events due to the larger excluded volume of the complex.

The first experimental condition we chose is at pH 4, in 3.6 M LiCl, as heparin molecules have the largest net force towards the trans side in this condition, resulting in maximum capture rate. Since Lectin binds 1:1 to Heparin, a 500 nM heparin solution and a 500 nM lectin solution were mixed in equal parts and left to incubate for about five minutes before sensing. At 200 mV, the results are presented in Figure 3.4.

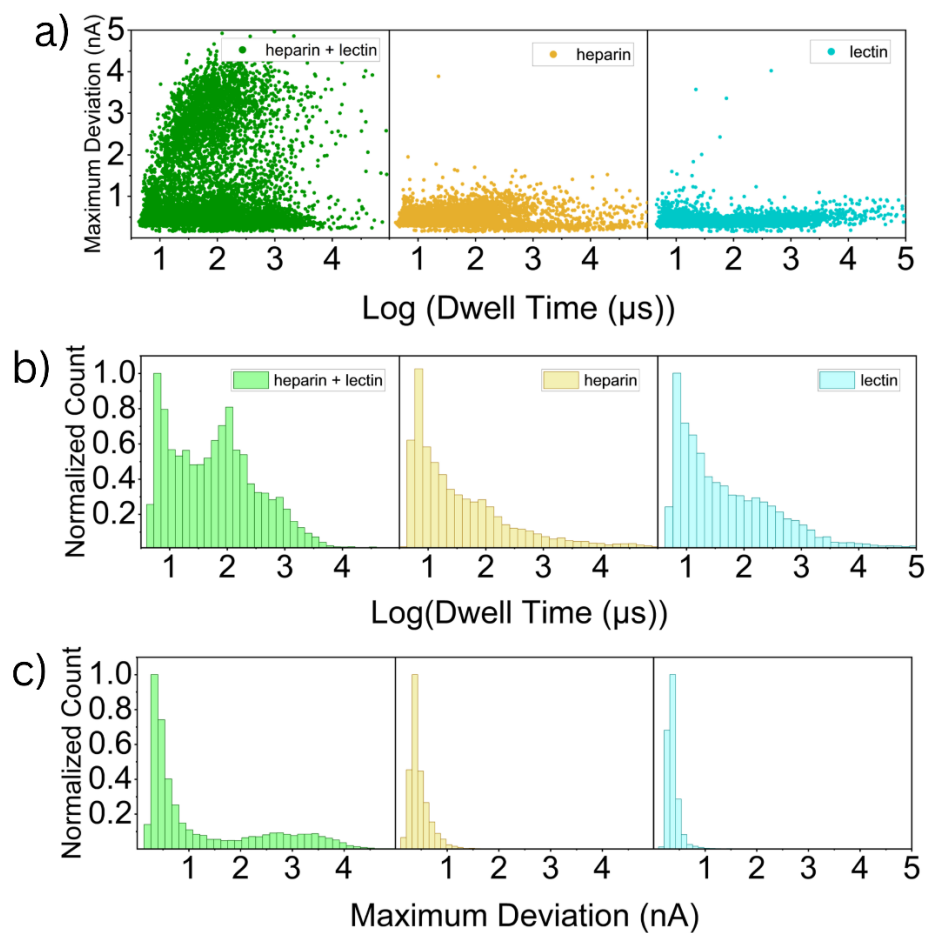


Figure 3.4 Scatter plots, histograms of dwell time and maximum deviation of heparin, lectin, and their mixture. a) Scatter plots of translocations of heparin and lectin mixture, and two molecules separately, in 3.6 M LiCl at pH 4, 200 mV, showing maximum deviation in nA

versus dwell time. Events shown 1200s of data acquisition for the mixture and lectin, 600s for heparin. $N \sim 8000$ for the mixture, $N \sim 6000$ for heparin, and $N \sim 3600$ for lectin; Experiments of the mixture and heparin were done in a $5.1 \text{ nm} \pm 0.2 \text{ nm}$ pore in a $17 \pm 2 \text{ nm}$ thick membrane, and the lectin experiment was done in a $10.8 \text{ nm} \pm 0.8 \text{ nm}$ pore in a $17 \pm 2 \text{ nm}$ thick membrane; **b)** normalized histograms of the dwell time distribution; **c)** normalized histograms of the blockage depth distribution.

We see that heparin signals are mostly shallower than 1 nA and faster than 10 μs , which fits the profile of our previous findings. Lectin signals exhibit similar events, shallow and fast, as one would predict, since the molecules are very small. However, in the lectin and heparin mixture, a new population with much deeper blockage (2 to 4 nA) appeared. The translocation time is also greatly increased to around 100 μs . Since neither heparin nor lectin alone exhibits similar characteristics, evidence suggests that this new population is the bound compound of heparin and lectin. As mentioned previously, heparin and lectin have a K_d of 6 nM. Although no direct investigation was done regarding the relationship between binding affinity and salt concentration between heparin and lectin, one study did find that the binding between heparin and another protein, thrombin, weakens as salt concentration increases[119]. However, considering the binding affinity between heparin and lectin is as high as it is, for the conditions stated above, the fraction of bound molecules should reach $\sim 90\%$.

Using a modelling tool developed by the lab and available online, called the signal generator[73], we can calculate the event signature produced by a variety of object shapes translocating a nanopore of a given geometry. Here, using a cylindrical molecule with a diameter of 4 nm, translocating through a 5 nm pore, generates a similar signal to what is experimentally observed. It further supports our interpretation that it is likely a complex of heparin and lectin. Indeed, as in Chapter 2, we found that heparin molecules most likely translocate in a folded fashion with an effective diameter of 2 nm, and lectin also has a diameter of $\sim 2 \text{ nm}$. Their complex, having a diameter of 4 nm, is certainly a possibility.

However, there is no other study of heparin-lectin-binding in similar conditions, and there is chemical information lacking regarding their molecular characteristics and interactions to further support this interpretation. We therefore repeated the experiment with 1 M KCl at pH 7, which is more similar to the conditions of Sandoval et al[117]. This time, we used an incubation condition of 2.5 μ M lectin and 250 nM heparin, creating an excess of lectin. A higher voltage was also adopted compared to the 3.6 M LiCl run, because in a previous voltage sweep conducted, it showed that the heparin capture rate in 1 M KCl is lower in comparison to higher molar salts at the same voltage (Figure 2.14). Unfortunately, there were no signs of binding this time. The heparin and lectin mixture produced near-identical populations as the lectin molecules.

In conclusion, we have demonstrated that solid-state nanopore systems can be a useful tool in studying carbohydrates. To better understand the sensing performance, the conformations and kinetics of heparin molecules translocating through a solid-state nanopore have been explored in the thesis. To improve the resolution of heparin events, slowing them down is necessary. Two methods, counterpressure and lectin binding, have been attempted in this outlook. From the results obtained, counterpressure does not have a significant effect on heparin translocation speed, but more work is needed to rule out this approach. Lectin and heparin seemingly bound and translocated as a complex in 3.6 M LiCl at pH 4, as deeper and slower populations emerged. However, this interaction was not present in 1 M KCl at pH 7. Future work should look at confirming these results and exploring how pH, salt concentration and voltage affect the stability of the lectin-heparin complex.

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